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EFFECTS OF URIDINE DIPHOSPHOGLUCURONIC ACID ON DRUG-INDUCED PORPHYRIN BIOSYNTHESIS

by

© JAMES TAYLOR KENNEDY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1969

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ABSTRACT

The aim of this thesis was to study the validity of a hypothesis formulated by Granick and Kappas (1967a) to explain the mechanism whereby Uridine Diphosphoglucuronic Acid (UDPGA) inhibits steroid-induced porphyrin biosynthesis in chick embryo liver cells. These workers suggested that UDPGA inhibits steroid-induced porphyrin biosynthesis by enhancing the glucuronidation and hence the inactivation of the steroid. We have shown that UDPGA not only inhibits porphyrin induction caused by steroids, but also by allylisopropylacetamide (AIA) and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC). This was not expected on the basis of the hypothesis of Granick and Kappas since AIA and DDC do not contain any of the groups which can be glucuronidated. On the basis of these experiments it appeared that the explanation offered by Granick and Kappas for the mechanism of UDPGA inhibition of porphyria-induction by steroids was incorrect. However, the results are not clear cut since it is possible that AIA and DDC are converted by the liver cells to metabolites containing groupings which allow them to be glucuronidated.

Incubation of cells for 12 hours with AIA (first incubation period) followed by removal of AIA by washing leads on reincubation (second incubation period) to an increase in porphyrin accumulation. If UDPGA was present during the

second incubation period, increased porphyrin accumulation was not observed. It is therefore clear that UDPGA is able to inhibit drug-induced porphyrin accumulation by mechanisms other than enhanced glucuronidation of a drug. Our results are compatible with a recent suggestion by Hickman et al. (1968) that the inhibitory effect of carbohydrates is due to interference with the coding of mRNA for δ-aminolevulinic acid synthetase.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. G. S. Marks, D. Phil. for his constant encouragement and able guidance, both of which contributed greatly to the completion of this thesis.

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CHAPTER I GENERAL INTRODUCTION

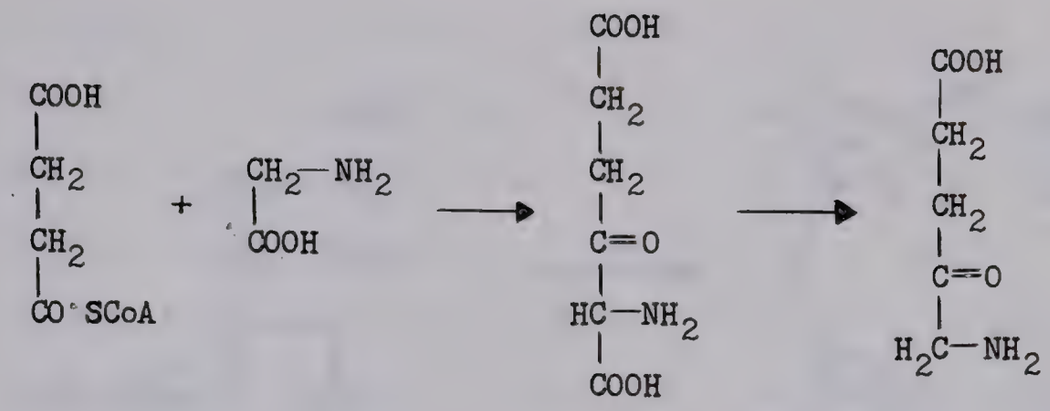
A. Distribution of Tetrapyrroles

The heme proteins constitute one of the most important classes of biological substances. They are widely distributed throughout nature, and in the form of hemoproteins and chlorophylls, are found in most forms of life, ranging from unicellular microorganisms to the higher animals. They are almost universally present in aerobic organisms, but may be found in some of the strict anaerobic bacteria and lactobacilli which obtain energy by the fermentation of organic substances. Among the heme proteins are found the respiratory proteins of animals, such as hemoglobin and myoglobin, and cellular oxidation catalysts of both animals and plants, such as the cytochromes, peroxidases, and catalases. Another heme protein is tryptophan pyrrolase which catalyzes the oxidation of tryptophan to formylkynurenine. As a class, the heme proteins are formed by conjugation of proteins with heme, an iron-porphyrin compound, which serves as the prosthetic group (West et al., 1966).

Porphyrins are also able to complex with metals other than iron. Examples of this are the chlorophylls, the major tetrapyrrole pigment in photosynthetic organisms, in which the tetrapyrrole is complexed with magnesium, and Vitamin B_{12} which contains cobalt. Complexes of porphyrin with copper, zinc, and manganese also occur in nature, for instance in urine, but they probably arise spontaneously since these complexes tend to form chelates easily, especially under mildly alkaline conditions (Lascelles, 1964).

B. Biosynthesis of Heme

By the early 1950's, it had been shown that the four nitrogen atoms and eight carbon atoms of heme had been derived from the amino acid, glycine. Through the use of radioactive isotopes by Shemin (1951), these eight carbon atoms were shown to be derived from the α-carbon atom of glycine, and while four of them were still attached to the nitrogen atom, four were not (Fig. 1B). Furthermore, it was found that the carboxyl group of glycine was not utilized for any of the carbon atoms of the porphyrin. It had also been demonstrated (Shemin, 1951) that a succinyl intermediate was the source of the remaining twenty-six carbon atoms of the porphyrin molecule. The next problem was to find a reasonable mechanism by which the succinyl intermediate and glycine combined so that the product supplied a precursor for pyrrole formation. Moreover, the mechanism had to explain how the α-carbon atom of glycine became detached from its carboxyl group as well as the distribution of the α-carbon atom of glycine in the porphyrin molecule. Shemin (Ciba Foundation, 1955, pp.10) suggested that the condensation of succinyl-CoA on the α-carbon atom of glycine to form α-amino-β-ketoadipic acid (Fig. 1A) would fulfill the above conditions. The compound formed, being a β-keto acid, would be readily decarboxylated. This would provide a mechanism by which the α-carbon atom of glycine becomes detached from its carboxyl group, and is therefore not found in the final porphyrin molecule. The product of decarboxylation would then be δ-amino-



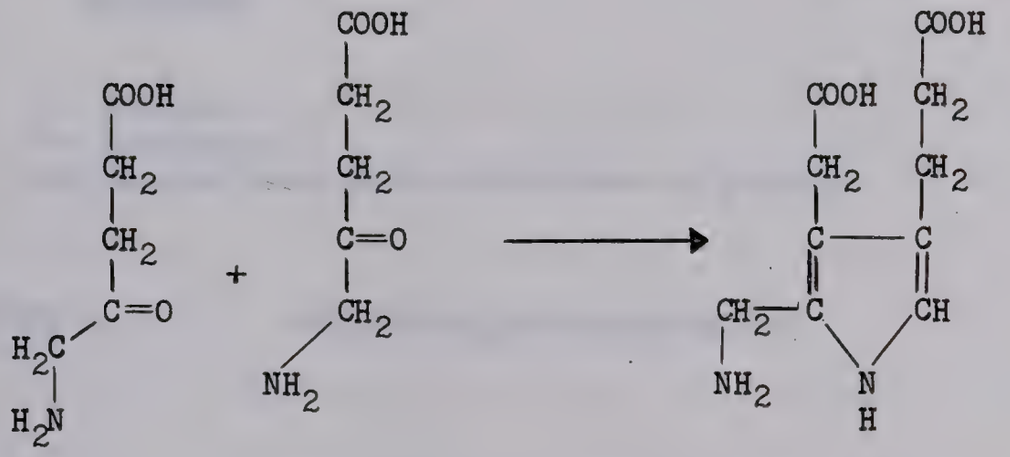
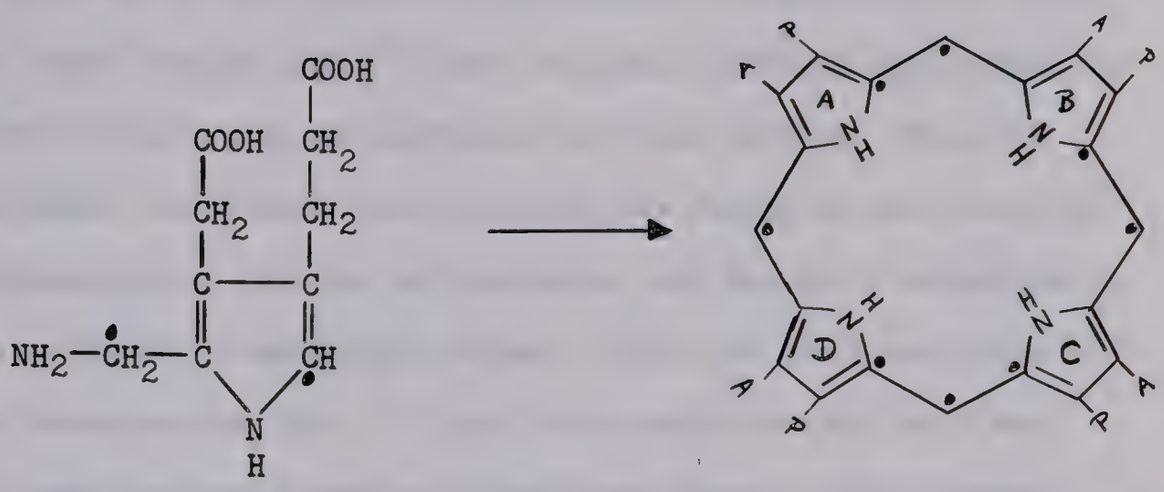


FIGURE 1A BIOSYNTHESIS OF HEME

levulinic acid which could condense to a pyrrole, porphobilinogen (Fig. 1A). Condensation of four moles of porphobilinogen would give a tetrapyrrole (uroporphyrinogen III) in which the α-carbon atoms of glycine are distributed in the positions previously observed in heme (Fig. 1B).



4 moles porphobilinogen

uroporphyrinogen III

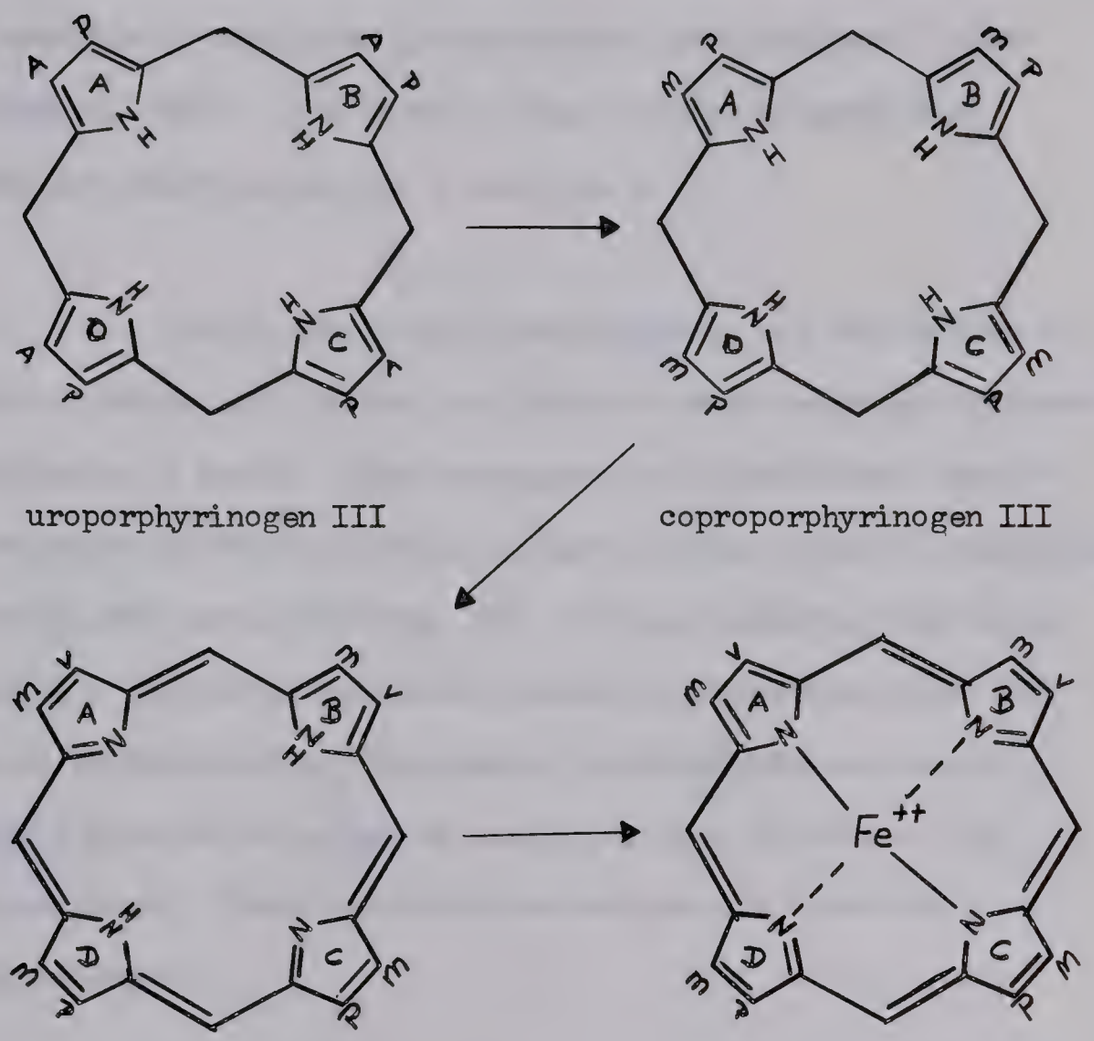
A = Acetate
P = Propionate
• = derived from the ∝-carbon atom of glycine.

FIGURE 1B BIOSYNTHESIS OF HEME CONT'D

To test this hypothesis, unlabelled δ-aminolevulinic acid was incubated with duck red blood cell hemolysate along with either ^{14}C-labelled glycine or ^{14}C-labelled succinate. The radioactivities of the heme samples isolated in these experiments were compared with those obtained from controls in which unlabelled δ-aminolevulinic acid was omitted. Thus, Shemin reasoned that if δ-aminolevulinic acid was an intermediate formed from the condensation of succinate and glycine, the radioactivity of the porphyrin formed in the controls should be greater than that of the porphyrin formed from ^{14}C-labelled succinate or ^{14}C-labelled glycine in the presence of unlabelled δ-aminolevulinic acid. The

heme formed in the presence of unlabelled δ-aminolevulinic acid was found to have less ^{14}C than the heme formed in experiments in which unlabelled δ-aminolevulinic acid was omitted. Thus, he concluded that δ-aminolevulinic acid was formed as the result of condensation of glycine and succinate, and was an intermediate in the porphyrin biosynthetic pathway. This was confirmed later by the demonstration that ^{14}C-labelled δ-aminolevulinic acid was utilized for heme formation (Shemin and Russell, 1953; Shemin, Russell and Abramsky, 1955).

Shemin (Schmid and Shemin, 1955) then investigated the next step in the synthesis of porphyrins. He incubated δ-aminolevulinic acid-5-^{14}C with a highly purified fraction from duck blood, and obtained labelled porphobilinogen. This porphobilinogen had twice the radioactivity of the labelled δ-aminolevulinic acid, and thus he concluded that two moles of δ-aminolevulinic acid were necessary to produce one mole of porphobilinogen, the next intermediate in the synthesis of porphyrins. Subsequent work of Bogorad (1958a; 1958b) showed that under the influence of two enzymes, uroporphyrinogen I-synthetase and uroporphyrinogen III-cosynthetase, four molecules of porphobilinogen condense with the loss of four molecules of NH_3 to form uroporphyrinogen III (Fig. 2), which is then decarboxylated by uroporphyrinogen decarboxylase yielding the intermediate coproporphyrinogen III (Mauzerall and Granick, 1958). Coproporphyrinogen III is then oxidized by the enzyme, coproporphyrinogen oxidase, to yield



A= acetic acid

P = propionic acid

M = methyl

V = vinyl

FIGURE 2 BIOSYNTHESIS OF HEME CONT'D

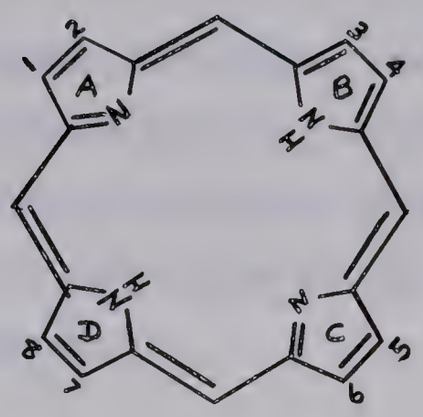
protoporphyrin IX. This oxidation involves the conversion of the two propionic acid groups in rings A and B of coproporphyrinogen III to vinyl groups and of the methylene bridges to methene bridges. The final step in the biosynthesis of heme is the enzymic incor-

poration of ferrous iron into protoporphyrin IX to give heme. This reaction is catalyzed by the enzyme ferrochelatase (Labbe and Hubbard, 1960). The various steps in this biosynthetic pathway are depicted in Fig. 1 and Fig. 2.

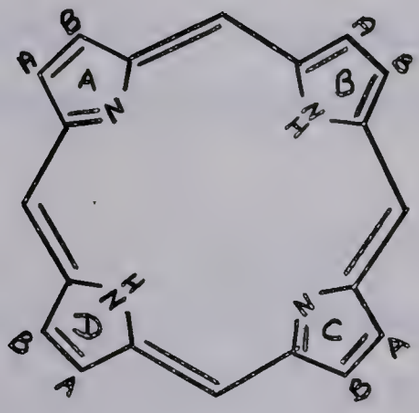
Uroporphyrin and coproporphyrin are derivatives of porphin in which each of the four pyrrole rings bears two different substituents, A and B. This arrangement of substituents leads to the existence of four different isomers, called series I, series II, series III and series IV (Fig. 3). Of these isomers, only those of series I and series III occur naturally; the others have been produced synthetically. Treatment of uroporphyrin and coproporphyrin with reducing agents results in the addition of six hydrogen atoms. These hexahydro-derivatives are known as porphyrinogens.

C. Diseases of Porphyrin Metabolism

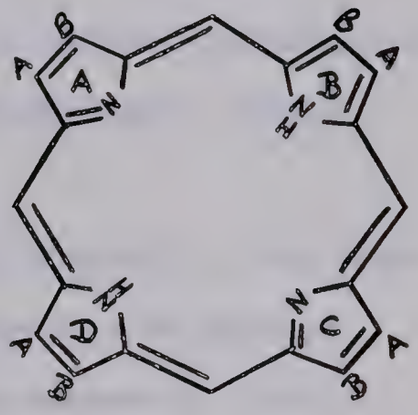
The erythropoietic system and the liver are important sites of porphyrin and heme formation. Heme formed in the erythropoietic system is utilized for hemoglobin formation while heme formed in the liver is incorporated into the enzymes cytochrome, peroxidase and liver catalase. The prophyrias are metabolic disorders of the porphyrin chemistry of the body in which the striking clinical expression is often the passage of a dark urine. In these diseases, porphyrins may accumulate in the bone marrow, particularly in the normoblastic nucleus, while in other cases,



porphin



Series I



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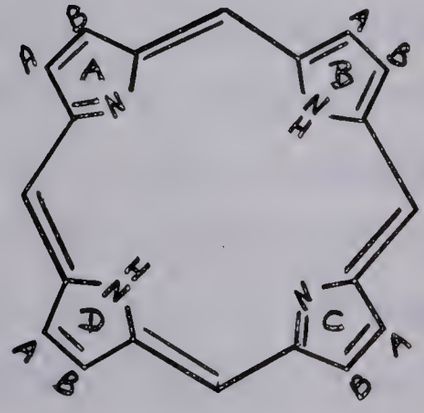

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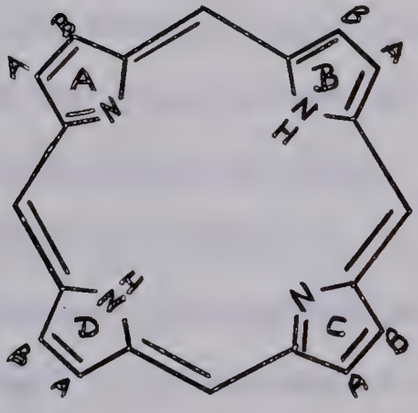


Series IV

For copro- A = CH_3, B = $CH_2CH_2CO_2H$

For uro- A = CH_2CO_2H, B = $CH_2CH_2CO_2H$

FIGURE 3 PORPHYRIN ISOMERS AND PORPHIN

porphyrins and porphyrin precursors accumulate in the liver. For this reason, the disorders of porphyrin metabolism have been classified as erythropoietic and hepatic porphyria respectively. These two classes have been further divided according to the mode of inheritance, clinical features observed, and the porphyrin or porphyrin precursors produced.

Two types of erythropoietic porphyria have been recognized viz., congenital erythropoietic porphyria and congenital erythropoietic protoporphyria (Goldberg and Rimington, 1962).

Congenital erythropoietic porphyria is a very rare disease of porphyrin metabolism in which there is an onset of photosensitivity in early life and complete absence of abdominal and neurological symptoms. The patient excretes excessive quantities of porphyrins, predominantly of the series I type. The spleen may be enlarged, the teeth brown and the urine is wine-red in colour. Anemia is usually present, and the disease is fatal. As the main porphyrin excreted in the urine is uroporphyrin I, a defect or absence of the enzyme, uroporphyrinogen III-cosynthetase, is probably involved. This disease is thought to be transmitted as a non sex-linked recessive character.

Congenital erythropoietic protoporphyria also manifests itself as a light-sensitive urticaria. The urinary excretion of porphyrins, porphobilinogen and δ-aminolevulinic acid is normal but levels of fecal porphyrins are raised. The

red cells contain increased amounts of protoporphyrin IX and coproporphyrin but, in contrast to congenital erythropoietic porphyria, there is no excess of uroporphyrin I in the blood or excreta. No evidence is forthcoming for hereditary transmission.

The hepatic porphyrias are divided into three major types viz., acute intermittent porphyria (Swedish type), cutaneous hepatic porphyria (South African type), and acquired hepatic porphyria. In the hepatic porphyrias, the defect seems to be limited to the liver cells (Goldberg and Rimington, 1962).

Acute intermittent porphyria is the most important of the group of porphyrin diseases and is clinically distinguishable from the others by the dominance of gastrointestinal and neurological symptoms and the absence of skin photosensitivity. In the acute and latent phases of this disease and often in states of remission, patients excrete in the urine excessive quantities of porphobilinogen and less frequently, of δ-aminolevulinic acid. These compounds are never excreted in excess in congenital porphyria and rarely in cutaneous hepatic porphyria. Among the factors believed responsible for precipitating the clinical manifestations of the disease, the administration of various drugs and various stress conditions are most important. The condition is believed to be inherited as a Mendelian dominant character, i.e. the individuals are heterozygous for an abnormal gene. There is, however, considerable variation in the degree of the expression of the character and in one family all grades can be

found from the acute case through latent porphyria to the apparently normal individual who excretes no porphobilinogen. The most frequent age of onset in this series was the third decade for females and the fourth decade for males.

Cutaneous hepatic porphyria is characterized by photosensitivity, acute attacks, neurological involvement, and may be induced by drugs. The predominant biochemical feature is the high fecal excretion of coproporphyrin and protoporphyrin. In contra-distinction to congenital erythropoietic porphyria, fluorocytes or fluorescing erythrocytes are absent from the blood. The mode of inheritance seems to be by means of a non sex-linked dominant gene.

Acquired hepatic porphyria clinically resembles cutaneous porphyria but is non-hereditary. In 1956, a large number of Turks consumed wheat intended for planting purposes which had been treated with the fungicide hexachlorobenzene, and acquired a hepatic form of porphyria. Photosensitivity, as shown by severe scarring of the hands and face, was the predominant clinical manifestation and this was accompanied by the excretion of uroporphyrin and coproporphyrin in the urine. The etiology of this disease was first suggested by Cam (1959) and this suggestion was supported by the finding that hexachlorobenzene readily induced a porphyria when fed to rats, guinea pigs, mice and rabbits (Ockner and Schmid, 1961; De Matteis et al., 1961). The porphyria observed in Turkey provides evidence for the occurence in man of

a purely acquired and not genetically predetermined form of hepatic porphyria.

D. Control of Tetrapyrrole Biosynthesis

Although it is clear that higher organisms have regulatory mechanisms for controlling the supply of intermediates for tetrapyrrole synthesis and for adjusting the synthesis in response to the environment, control mechanisms have previously been studied mostly in bacteria. The high rate of synthesis achieved by microorganisms and the fact that they can grow in defined media in environments that can be varied experimentally have undoubtedly been major reasons for their exploitation. From experimental evidence, two control mechanisms have been firmly established (Lascelles, 1964).

i) feedback control of enzyme action, in which the end product inhibits the enzyme catalyzing the first step leading specifically to the end product; and

ii) feedback control of enzyme formation (repression), in which the end product acts as a repressor of enzyme formation and in many instances synthesis of all the enzymes of the pathway is prevented (coordinate repression). Repressibility is under genetic control since mutants have been isolated in which enzyme synthesis is no longer repressed by the end product.

An example of end product inhibition was observed by Lascelles (1956) in the photosynthetic bacterium Rhodopseudomonas

spheroides. When this bacterium was grown in an iron-free medium, Lascelles reported that coproporphyrin III was excreted into the medium in concentrations up to 200 µm-moles/ml. When small amounts of iron were added to the culture, porphyrin production decreased substantially and Lascelles suggested that traces of iron combined with porphyrin to produce heme, which in turn inhibited the first enzyme in the pathway, viz., δ-aminolevulinic acid synthetase. This suggestion was later confirmed by the demonstration of Burnham and Lascelles (1963) using partially purified cell extracts from Rhodopseudomonas spheroides that hemin inhibits the action of δ-aminolevulinic acid synthetase. London, Bruns and Karibian (1964) reported that the incorporation of tracer glycine into the heme of hemoglobin occurring in rabbit erythrocytes was decreased by the addition of heme and this was interpreted as reflecting feedback inhibition by heme on δ-aminolevulinic acid synthetase.

Granick and Urata (1963) found that whereas guinea pig liver δ-aminolevulinic acid synthetase activity could barely be detected, considerable quantities of the other enzymes of the biosynthetic chain were present, suggesting that porphyrin synthesis was limited by the activity of δ-aminolevulinic acid synthetase. When porphyria-inducing drugs were fed to the guinea pigs, the level of this enzyme increased approximately forty fold leading to porphyrin accumulation. Similarly, when a porphyria-inducing chemical was added to intact chick embryos, Granick found

that the δ-aminolevulinic acid synthetase activity increased eight fold in the chick liver mitochondria. Using the chick embryo liver cell culture method, Granick found that the increased δ-aminolevulinic acid synthetase activity induced by porphyria-inducing drugs was abolished by agents that prevented DNA replication or protein synthesis. Granick therefore concluded that the inducing chemicals caused an increase in the synthesis of δ-aminolevulinic acid synthetase, rather than either activation of an inactive enzyme, or decrease in enzyme destruction. To quote Granick (1966): "On the basis of these findings, it is surmised that the synthesis of δ-aminolevulinic acid synthetase is repressed in normal liver and that the inducing chemical interferes with the repressor mechanism".

The mechanism of feedback repression involves a specific repressor molecule that is considered to interact with the operator gene and thus prevent the structural gene from being decoded into mRNA (Fig. 4; Jacob and Monod, 1961). The repressor is considered to be a protein (called the aporepressor) to which is attached the corepressor, heme. The inducing chemicals are postulated to compete with the corepressor for the corepressor site, resulting in an inactive repressor. Thus, the operator gene is not repressed by the repressor and therefore allows the structural gene to produce mRNA which results in the formation of δ-aminolevulinic acid synthetase. Because δ-aminolevulinic acid synthetase is the rate-limiting enzyme in the biosynthetic pathway, excessive amounts of porphyrins are produced. Since the

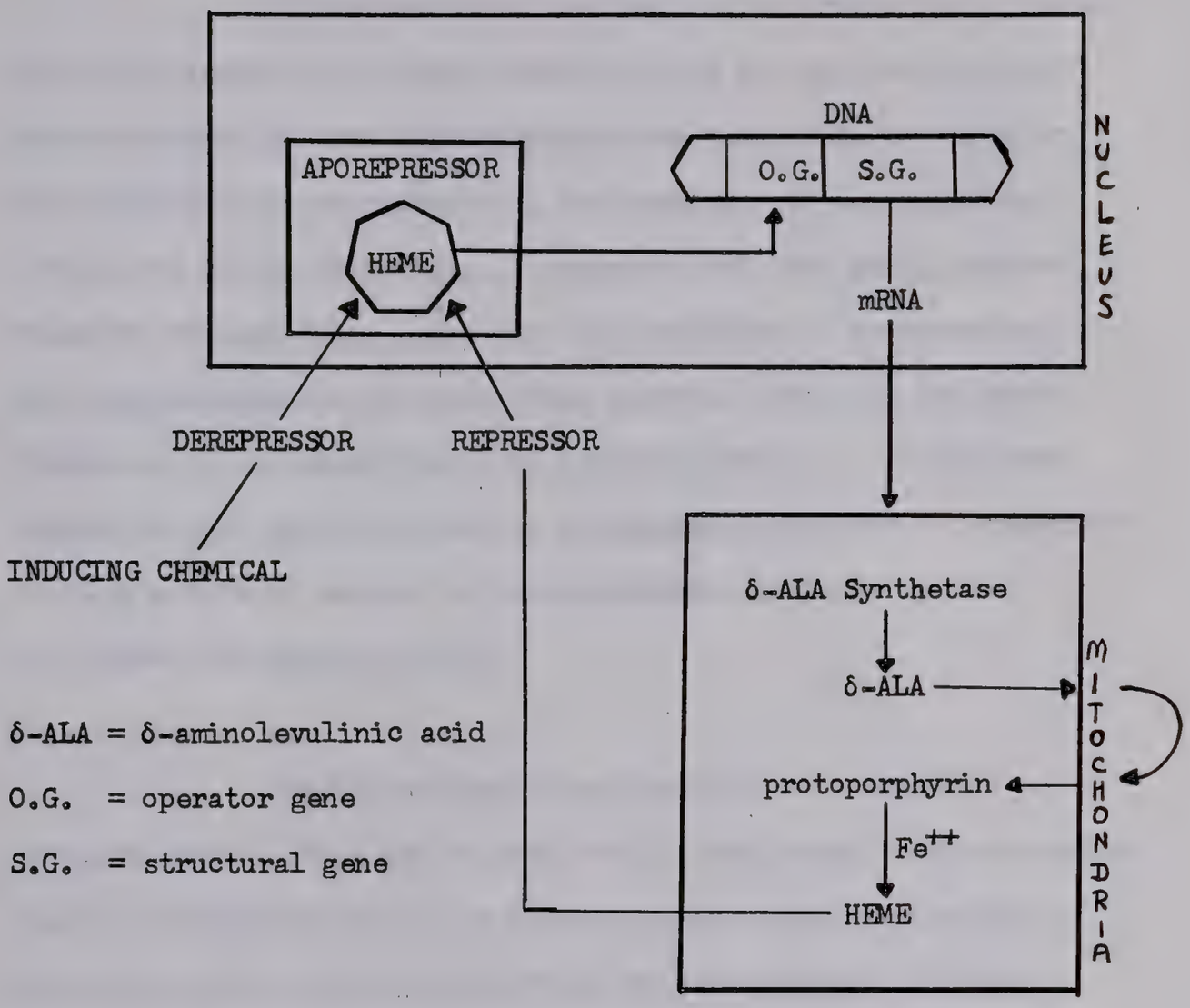


FIGURE 4 MECHANISM OF FEEDBACK REPRESSION

inducing chemicals compete for the corepressor site, increasing the concentration of the corepressor (heme) in the presence of the inducing chemical should result in decreased induction. Experimental evidence has shown that increasing the concentration of the corepressor heme, in the presence of an inducing chemical, does in fact result in a decrease in the amount of porphyrins formed (Granick, 1966).

Of the reactions involved in the production of heme, only the formation of δ-aminolevulinic acid and the conversion of protoporphyrin IX from coproporphyrinogen take place in cellular particulates (the mitochondria), the remainder of the reactions take place in the cytoplasm. It appears that this acid, synthesized in mitochondria, leaks into the cytoplasm to be converted into coproporphyrinogen, which then migrates back into the mitochondria, to be transformed into protoporphyrin IX. It had been suggested that this localization of enzymes might permit permeability to play a part in control of protoporphyrin synthesis by the cell (Sano and Granick, 1961).

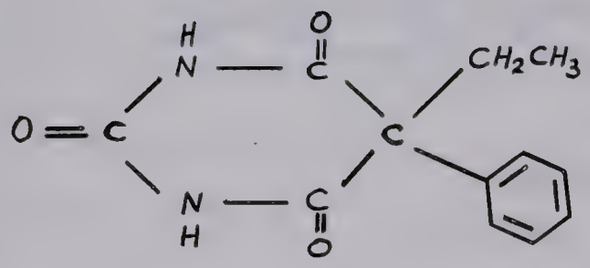
Hepatic porphyria is inherited as a Mendelian dominant trait. This can be most readily explained on the assumption that the defective gene is an operator gene which is mutated so that it is repressed with difficulty by the repressor (Granick, 1966). For this reason, small amounts of inducing chemicals which would cause no detectable change in porphyrin metabolism in normal individuals precipitates an attack of porphyria in individuals with the inherited defect.

E. Relationship Between Structure and Porphyria-Inducing Activity

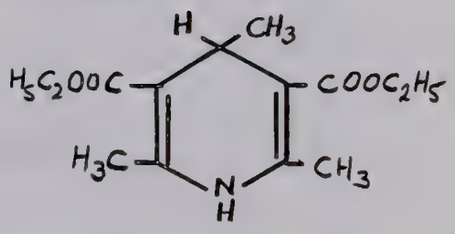
A number of chemicals of diverse structure are known that cause a porphyria in animals. This porphyria mimics the inheritable human disease, hepatic porphyria, in the urinary

excretion of porphyrins and their precursors, porphobilinogen and δ-aminolevulinic acid. These chemicals have been classified into four categories by Granick (1966). These are the barbiturates, the collidines, the sex steroids, and a miscellaneous class which contains such compounds as griseofulvin and hexachlorobenzene (Fig. 5). These chemicals are potent inducers of porphyrins in chick embryo liver cell culture systems and may precipitate a severe acute attack of porphyria when administered to patients with the inherited genetic trait for this disease.

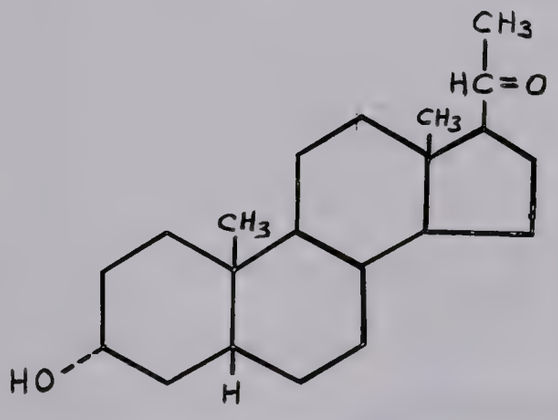
Granick and Kappas (1967a) found that a number of metabolites of the 5-β (A:B Cis) C_{19} and C_{21} sex steroid hormones (Fig. 6) were very potent inducers of porphyria in the chick embryo liver cell culture system. These metabolites, such as pregnanolone and etiocholanolone, which had been generally considered to represent biologically inert degradation products of hormone metabolism were active in concentrations as low as 10^{-6} to 10^{-8} molar. Several of them are produced in significant amounts daily from the metabolic conversions of such precursor hormones as testosterone and progesterone. Thus, Granick suggested that they may play a role in heme biosynthesis in liver cells through the regulation of the formation of δ-aminolevulinic acid synthetase. These compounds are also active in inducing porphyrin formation in erythroid cells. In contrast, foreign compounds, such as allyl-isopropylacetamide (AIA) and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) (Fig. 5 and Fig. 7), which have



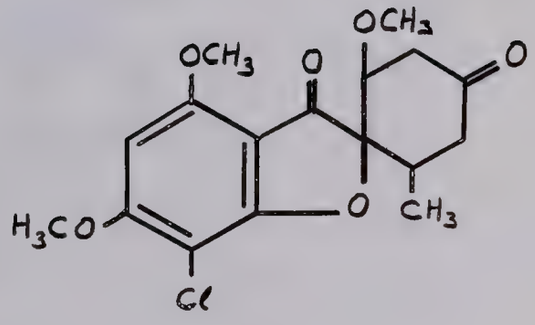
phenobarbital



3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC)

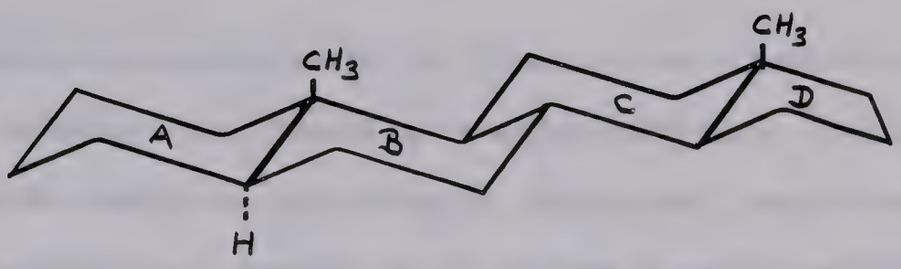


5β-pregnane-3α-ol-20-one (pregnanolone)

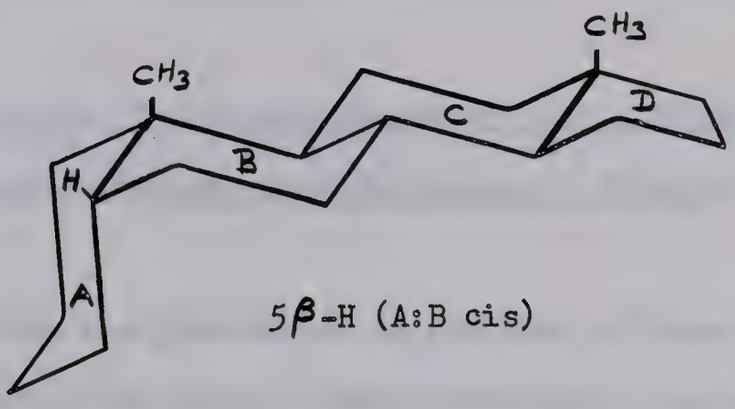


griseofulvin

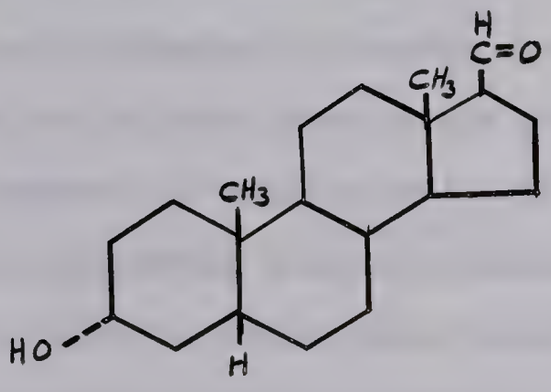
FIGURE 5 CLASSES OF PORPHYRINOGENIC CHEMICALS



5α-H (A:B trans)



5β-H (A:B cis)



5β-androstane-3α-ol,17-one
(etiocholanolone)

FIGURE 6 SEX STEROID HORMONES

strong porphyria-inducing action in liver, do not stimulate excess porphyrin or hemoglobin formation in erythroid cells. The fact that steroids, which are physiological substances, are active in both the erythroid and hepatic cells further suggests that they might be involved in the *in vivo* heme control mechanism.

F. Effect of Uridine 5'-diphosphoglucuronic Acid (UDPGA) on Induction of δ-Aminolevulinic Acid Synthetase by Steroids

When the glucuronide derivatives of these steroids were tested, Granick and Kappas (1967b) noted that they were completely devoid of porphyria-inducing activity. Induction of δ-aminolevulinic acid synthetase by steroids was inhibited in the chick embryo liver cell culture system by the addition of UDPGA but by no other component of the glucuronic acid pathway. This effect was viewed as reflecting enhanced conversion of the active free steroid to the inactive steroid-glucuronide by the enzyme UDP-glucuronyl transferase, which is present in small amounts in the chick embryo liver (Dutton, 1966; Fig. 8). On the basis of the repressor-operator control mechanism, steroids would induce δ-aminolevulinic acid synthetase by competing with heme for a binding site on the aporepressor protein, thus preventing repression of the operator gene. To do this, the steroids would have to be in the free and not the glucuronidated form. However, the steroid glucuronides may also be hydrolyzed back to the active form by the enzyme, β-glucuronidase. Thus, any process which led

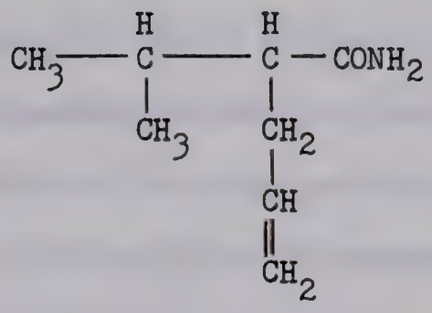


FIGURE 7 STRUCTURE OF ALLYLISOPROPYLACETAMIDE (AIA)

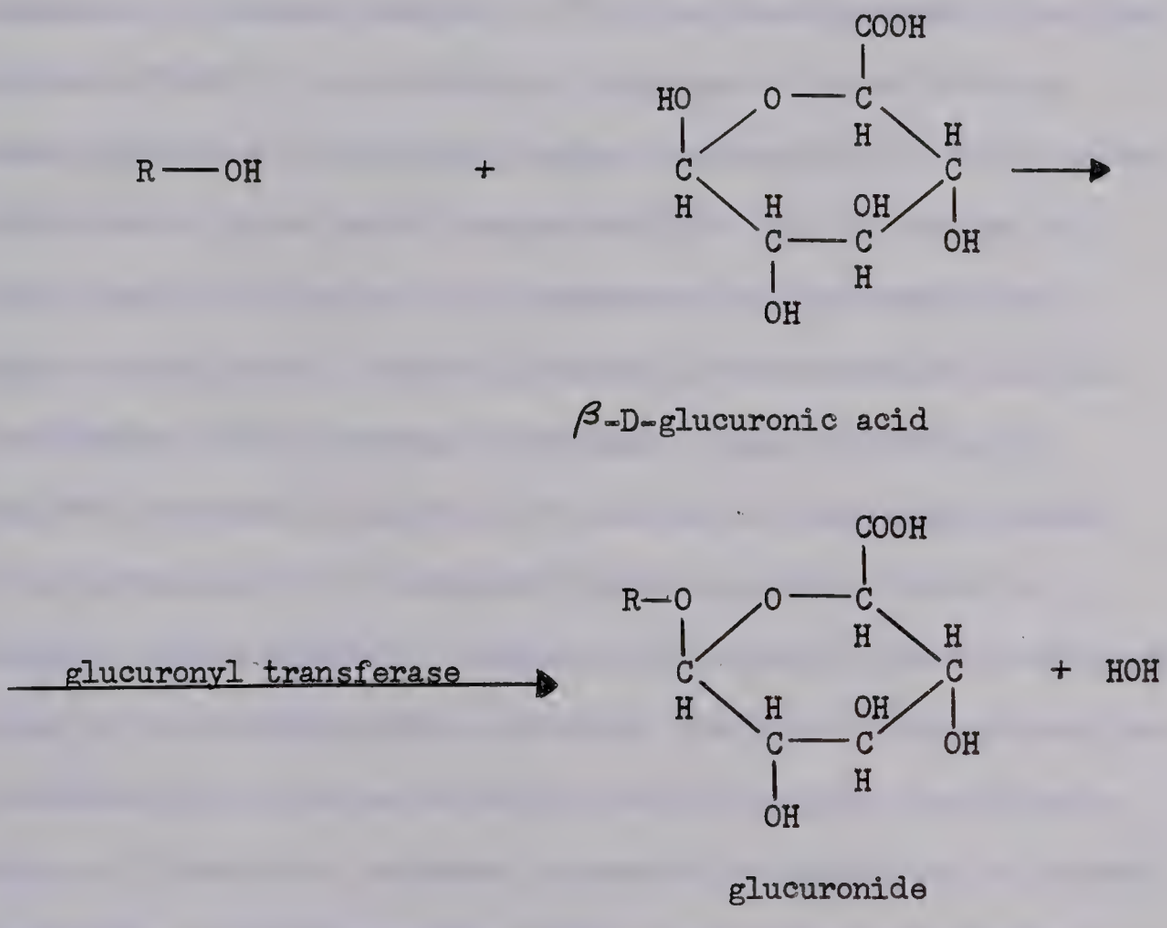


FIGURE 8 CONJUGATION WITH GLUCURONIC ACID

to (a) enhanced intracellular hydrolysis of steroid glucuronides, (b) impaired glucuronidation of the steroids, or (c) steroid production in amounts exceeding the body's capacity to dispose of them would lead to induction of δ-aminolevulinic acid synthetase and, as a consequence, to accumulation of porphyrins (Granick and Kappas, 1967a).

The feeding of large amounts of glucose to animals has been found to inhibit the induction of δ-aminolevulinic acid synthetase by certain chemicals. This had been referred to as the 'glucose effect'. The inhibitory influence of added UDPGA on steroid induction of porphyria raises the possibility that glucose inhibition of induction of δ-aminolevulinic acid synthetase in animals may be accounted for by conversion of the administered sugar to UDPGA which leads to enhanced glucuronidation, and thus inactivation of the inducing substances. Thus, according to Granick's hypothesis, the role of steroids as provocative agents in the pathogenesis of hereditary hepatic porphyria might be dependant on the quantity of unconjugated steroid inducers which gained access to the inducing site or sites in the liver. Any process such as starvation or glucose restriction which impaired the glucuronidation of steroids or enhanced intracellular hydrolysis of steroid glucuronides by β-glucuronidase might lead to the accumulation of inducing steroids in liver cells in concentrations sufficient to stimulate further excess porphyrinogenesis in subjects carrying the genetic lesion for this disease. Excessive production of

inhibitors of UDP-glucuronyl transferase could also lead to the accumulation of high concentrations of inducing steroids in liver cells. In this respect, it is of interest that certain potent porphyria-inducing 5β-H steroids themselves, such as pregnandiol, may inhibit the activity of UDP-glucuronyl transferase in liver (Kappas and Granick, 1967).

G. Statement of the Problem

The purpose of this thesis was to investigate Granick's observation that UDPGA inactivates the porphyria-inducing steroids and to try to elucidate the mechanism by which this occurs. In the first series of experiments, it was decided to investigate the effects of UDPGA on porphyrin accumulation induced in chick embryo liver cells by AIA and DDC. If Granick's explanation for the action of UDPGA was correct viz., that it caused enhanced glucuronidation of the inducing drug, then it was anticipated that it should have no effect on porphyria-induction by AIA and DDC. This follows from the fact that AIA and DDC do not contain groups with which glucuronic acid could combine. This work is reported in Chapter III of this thesis.

In the second series of experiments, chick embryo liver cells were incubated with a porphyria-inducing drug for 12 hr. At this time, the drug was removed by washing and the cells were reincubated for 5 - 8 hr whereupon an increase in porphyrin accumulation occurred due to the presence of mRNA for

δ-aminolevulinic acid synthetase and δ-aminolevulinic acid synthetase itself. The action of UDPGA was studied during the reincubation period to see if UDPGA was effective in preventing porphyrin accumulation in the absence of the drug. This work is reported in Chapter IV of this thesis.

CHAPTER II MATERIALS AND METHODS

A. Protein Determination

The method used was that of Lowry et al. (1951) as modified by Miller (1959). A standard solution of Bovine Serum Albumin (Mann Research Laboratories; 100% purity; 200 µg/ml) was prepared by dissolving 20 mg Bovine Serum Albumin (BSA) in 100 ml of double-distilled water. The standard solution was diluted with double-distilled water to make solutions of 50, 100, and 150 µg BSA/ml. Duplicate 1 ml aliquots of each sample were used to make the standard curve.

Reagents

i) Diluted Folin-Phenol Reagent: This was prepared by mixing 5 ml of Folin-Phenol Reagent (BDH) with 50 ml of double-distilled water;

ii) Copper Reagent: This consists of:

a) 1% copper sulphate (1 ml);

b) 2% sodium potassium tartarate (1 ml);

c) 10% sodium carbonate in 0.5 molar NaOH (20 ml).

Aliquots of the protein solution (1 ml) were mixed with aliquots of the copper reagent (1 ml) and allowed to stand at room temperature for 10 min. Diluted Folin-Phenol Reagent (3 ml) was then added to each sample, the mixture shaken thoroughly, and placed in a water-bath at 56° for 10 min. The optical density of the

solutions was then read at 540 mμ on a Unicam Spectrophotometer (Model SP 600) against distilled water. The following results were obtained:

TABLE I PROTEIN STANDARD CURVE

Tube No.	Concentration Protein (μg/ml)	Optical Density	Corrected Optical Density	Mean Value
Blank	0	0.020	-	-
1	50	0.136	0.116	0.126
2	50	0.157	0.137	
3	100	0.260	0.240	0.238
4	100	0.257	0.237	
5	150	0.367	0.347	0.331
6	150	0.335	0.315	
7	200	0.426	0.406	0.371
8	200	0.356	0.336	

The results were plotted as μg protein/ml versus optical density ($x10^{-2}$) to give the standard curve (Fig. 9A).

B. Porphyrin Determination

This method is based on the method of Talman (1958) as modified by Schwartz. Coproporphyrin I methyl ester (556 μg) was weighed out on an electrobalance. The sample was then transferred to a 100 ml volumetric flask, 7.5N HCl (1 ml) added, and the sample was hydrolyzed overnight to yield coproporphyrin I. The 556 μg of the ester is equivalent to 513 μg of free copro-

porphyrin I. The volumetric flask was filled to 100 ml by the addition of a mixture of 1M $HClO_4$ and 98% ethanol (1:1 v/v) to give a final concentration of 513 µg coproporphyrin I/ 100 ml. A series of dilutions was prepared with a mixture of $HClO_4$:ethanol (1:1 v/v) and read in the Turner Fluorometer (Model 110) fitted with a 405 mu band pass primary filter and a Wratten No.25 (595 mµ) sharp cut secondary filter. The blank used consisted of a mixture of $HClO_4$:ethanol (1:1 v/v; 4 ml). The following results were obtained:

TABLE II PORPHYRIN STANDARD CURVE

Sample No.	Concentration Coproporphyrin I (µg/100 ml)	Fluorometer Reading
1	0.0256	5.5
2	0.128	23.0
3	0.256	50.5
4	0.347	59.0
5	0.513	91.0

These results were then plotted in Fig. 9B.

C. Technique of Primary Liver Cell Culture

a) Composition of Solutions Used:

1) Calcium and Magnesium free Eagle's Medium: The following

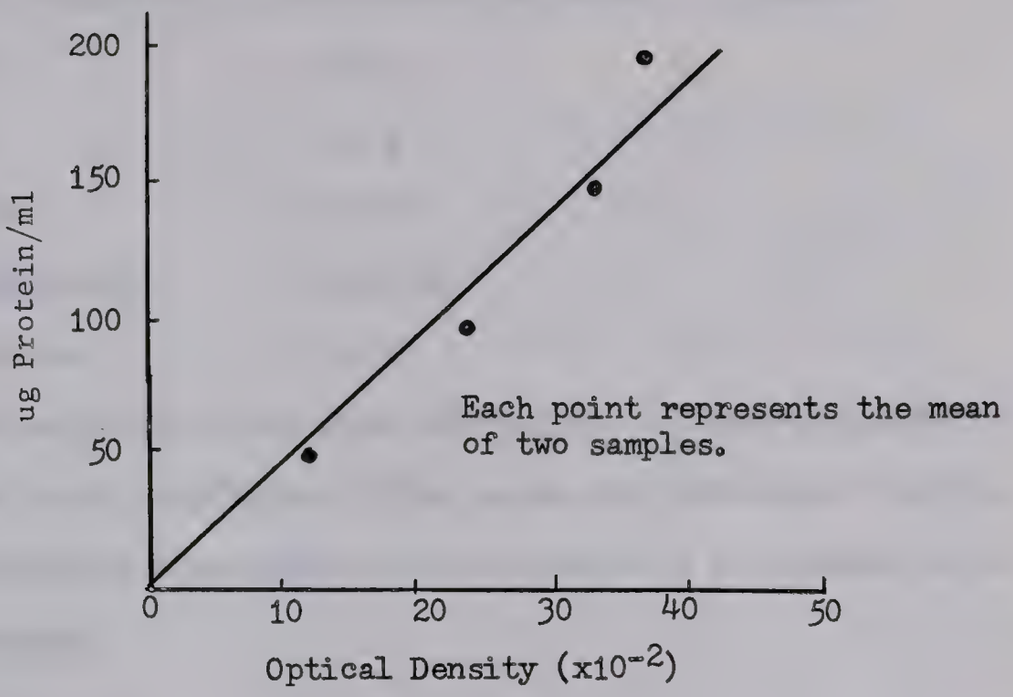


FIGURE 9A PROTEIN STANDARD CURVE

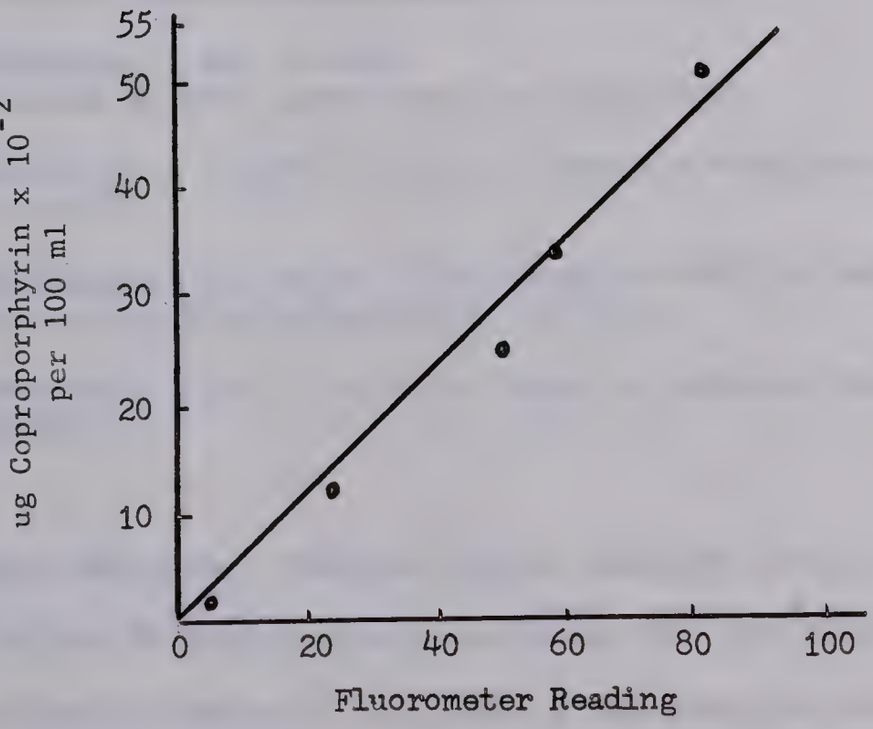


FIGURE 9B PORPHYRIN STANDARD CURVE

salts were dissolved in double-distilled water (1 liter):

i) NaCl - 6.8 g

ii) KCl - 0.4 g

iii) $NaHCO_3$ - 2.2 g

iv) $NaH_2PO_4 \cdot H_2O$ - 0.125 g

v) Dextrose - 1.0 g

The pH was adjusted to 6.8 using dilute HCl, and the solution was sterilized using a millipore filter apparatus (pore size - 0.45u). In the subsequent discussion, this solution will be referred to as Eagle's Medium.

2) Culture Medium: The culture medium consists of the following:

i) Earle's Basal Medium without Phenol Red (100 ml) (Microbiological Associates, Cat. #12-104);

ii) Fetal Bovine Serum (10 ml) (Microbiological Associates, Cat. #14-413);

iii) Glutamine (1 ml; 200 mM) (Microbiological Associates, Cat. #17-605F)

iv) Penicillin G (10,000 U in 0.1 ml sterile distilled water) (Ayerst);

v) Streptomycin (12 mg in 0.2 ml sterile distilled water) (Strepolin, Glaxo-Allenburys; 33% w/v);

vi) Mycostatin (2,000 U in 0.2 ml sterile distilled water) (Squibb).

3) Trypsin Solution: Prepared trypsin solution (5 ml; 2.5% in normal saline; Microbiological Associates, Cat. #17-160F) was added to Eagle's Medium (5 ml). This preparation was used immediately.

b) Procedure for Liver Cell Culture:

Five livers from 17-day old chick embryos were washed three times by transferring them through three small petri dishes (60 x 15 mm) containing Eagle's Medium. The livers were transferred to another petri dish containing trypsin solution. The tissue was minced with a scissors and razor blade, and incubated for 30 min in a serological bath at 37°. During this time, the tissue fragments were gently sucked in and out of a sterile Pasteur pipette until a homogeneous creamy consistency was obtained. The suspension was transferred to a large centrifuge tube, and the debris and undigested particles allowed to settle out for 5 min. The supernatant was transferred to a second centrifuge tube and spun for 5 min at 1,000 x g. The supernatant was discarded, and the cells resuspended in culture medium (9 ml).

Aliquots of the cell suspension (0.2 ml) were placed in petri dishes containing culture medium (4 ml) and the cells were allowed to incubate at 37° in 95% air: 5% CO_2 for 24 hr. The medium was replaced with fresh medium (4 ml). The drugs, dissolved in appropriate solvents, were then added and the incubation allowed to proceed for the appropriate time. Controls in which the solvent alone was used were always run at the same time. The porphyrins were then extracted by a procedure described below.

D. Counting of the Liver Cells

The cell suspension was diluted 1:20 v/v with normal saline, transferred to a hematocytometer and the number of cells counted with a microscope. From the results of this count it was calculated that the average number of cells added to each petri dish was 1.3×10^6/0.2 ml cell suspension.

E. Observation of Fluorescence Intensity by Microscopy

After the supernatant solution was removed and the cells washed with Eagle's Medium (2 ml), the cells were examined for the presence of porphyrins by means of a fluorescence microscope (Fig. 10). The Zeiss Standard Universal Fluorescence Microscope was used with a Number 1 excitor filter and a combination of Number 53 and 44 barrier filters. The fluorescence intensity was graded on a scale from 1 to 4 on the basis of the following criteria:

+1: some colonies fluoresce partially;

+2: most colonies fluoresce partially;

+3: most colonies fluoresce intensely;

+4: all colonies fluoresce intensely.

The grading scale was based on the work of Granick (1966).

F. Phase Contrast Microscopy

Phase contrast microscopy was used to determine the condition of the cell colonies. The criteria for healthy cells

were as follows: 1) finger-like cytoplasmic projections at the periphery of the colonies; 2) clear cytoplasm with abundant nuclei; 3) abundant growth (Fig. 11). Retraction of the cytoplasmic projections, rounding up of the parenchymal cells, and detachment of the cells from the glass were recognized as inhibition of growth, injury, and death respectively (Granick, 1966).

G. Extraction of the Porphyrins

a) From cells: After removing the medium from the dishes, the cells were observed by means of fluorescence and phase contrast microscopy. The petri dishes were cooled to 4° and 1M $HClO_4$: ethanol (1:1 v/v; 4 ml) was added to each dish. After 5 min, the solvent was removed and replaced by fresh solvent (4 ml). After 5 more min, the solvent was removed and combined with the first extract. The porphyrin content of the combined extracts was determined in the fluorometer as follows. Using the porphyrin standard curve, the fluorescence reading is converted to ug coproporphyrin x 10^{-2}/ 100 ml extract. In certain cases the extract had to be diluted with 1M $HClO_4$: ethanol (1:1 v/v) before it could be read in the fluorometer.

b) From medium: The medium from the petri dish was combined with Eagle's Medium (2 ml) used to wash the cells, and stored frozen until assayed.

The thawed solution was poured into a separatory

funnel (60 ml). Diethyl ether (10 ml) was added, along with glacial acetic acid (0.2 ml). The mixture was shaken whereupon an emulsion formed. The emulsion and the ether layer, containing the porphyrin, were retained while the aqueous layer was discarded. The ether layer and emulsion were centrifuged at 1,100 x g for 5 min to sediment the emulsion, which collected at the interface of the ether-water layer. The ether layer was then transferred to a separatory funnel with a Pasteur pipette and 1M $HClO_4$ (2 ml) was added. The porphyrin was extracted into the 1M $HClO_4$. The 1M $HClO_4$ layer was then added to 98% ethanol (2 ml) and the fluorescence of the solution determined in the fluorometer after appropriate dilution if necessary.

Using the standard curve, porphyrin concentration was then determined. This value was added to the value of porphyrin obtained from the cells, and the combined value gave the total amount of porphyrin accumulation in the cells and medium.

H. Protein Determination

Following the extraction of porphyrins from the cells, the cells were rinsed successively with 95% ethanol and an ethanol: ether mixture (3:1 v/v), and allowed to dry in a fume hood. They were stored at 10° until used.

1N NaOH (4 ml) was added to each dish and the cells

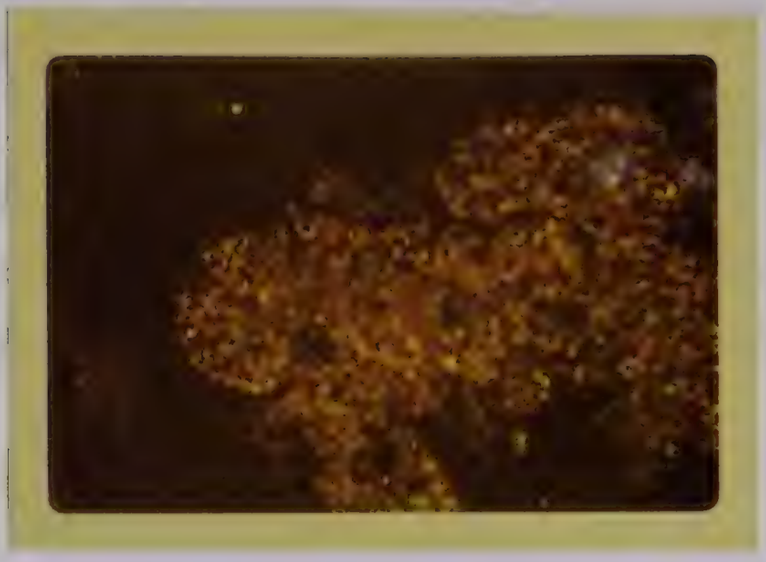


FIGURE 10 PHOTOGRAPH OF FLUORESCING CELLS

FIGURE 11 PHOTOGRAPH OF CELLS AS SEEN UNDER PHASE CONTRAST MICROSCOPY

scraped free of the glass surface by means of a scalpel blade. The fluid was transferred to test tubes and boiled in a water bath for 10 min. The solution was poured into petri dishes and neutralized with 1N HCl (approx. 3.8 ml) using pH paper as the indicator. The neutralized solution (1 ml) was used for determination of protein content by the procedure described above.

CHAPTER III STUDIES OF THE EFFECTS OF URIDINE DIPHOSPHO-GLUCURONIC ACID ON DRUG-INDUCED PORPHYRIN FORMATION

Introduction

Induction of δ-aminolevulinic acid synthetase by steroids is inhibited by UDPGA and this effect is regarded by Granick and Kappas (1967a) as reflecting enhanced conversion of the active free steroid to the inactive steroid-glucuronide. If this explanation is correct it is likely that UDPGA should have no effect on porphyria-induction by AIA and DDC. This follows from the fact that AIA (Fig. 5) and DDC (Fig. 7) do not contain groups with which glucuronic acid could combine. We have therefore investigated the effect of UDPGA on porphyria-induction by AIA and DDC.

Experimental

i. Relationship Between Dose of Drug and Porphyrin Accumulation

Dose-response curves for pregnanolone, AIA and DDC were prepared in order to determine a concentration of these chemicals which would give an adequate response in the chick embryo liver cell culture system. The cell colonies were prepared as mentioned previously and allowed to incubate for 24 hr. The culture medium was then removed and replaced by fresh medium (4 ml). The chemicals under investigation were then added to the cells by means of λ pipettes. AIA and DDC were dissolved in ethanol (95%) for purposes of administration, and pregnanolone was dissolved in propylene glycol. The maximum amount of solvent added to each

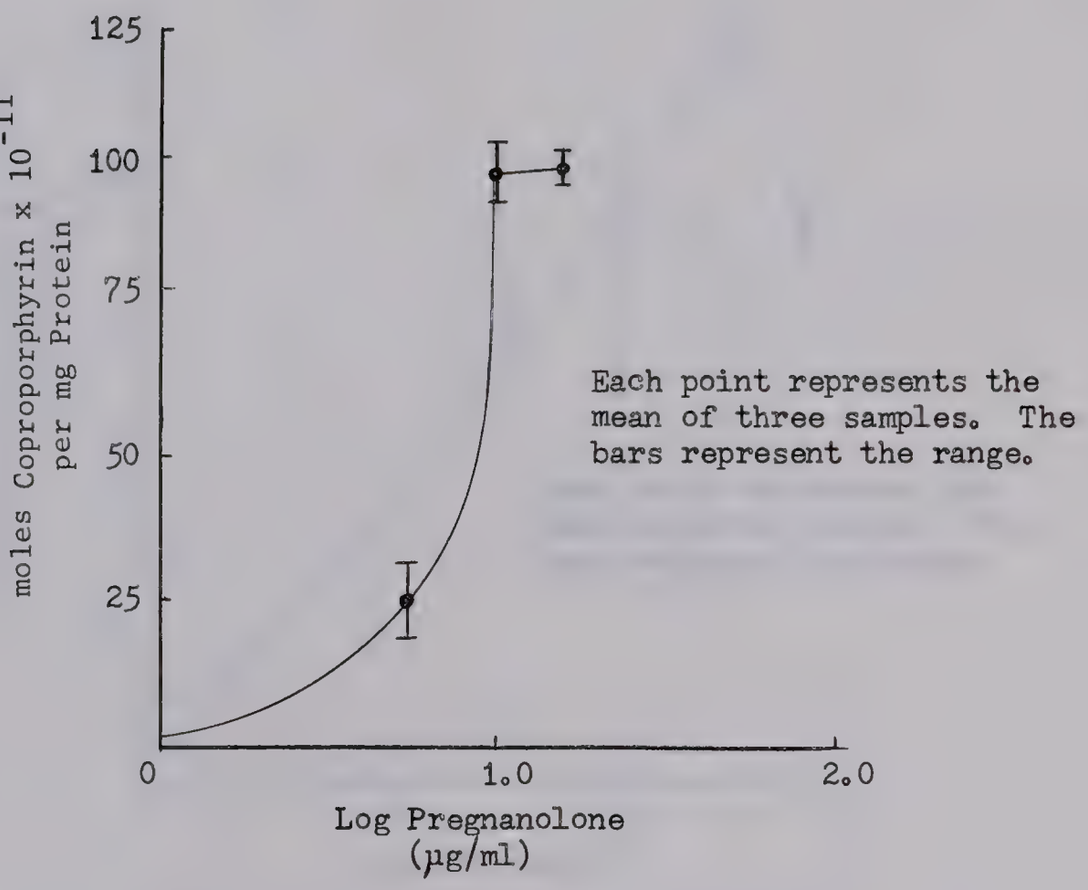


FIGURE 12 RELATIONSHIP BETWEEN DOSE OF PREGNANOLONE AND PORPHYRIN ACCUMULATION

petri dish was 5 λ. After the addition of the chemicals, the culture dishes were returned to the incubator for approximately 24 hr. The porphyrin content of the cells and media was then determined, as well as the protein content per dish. The curves

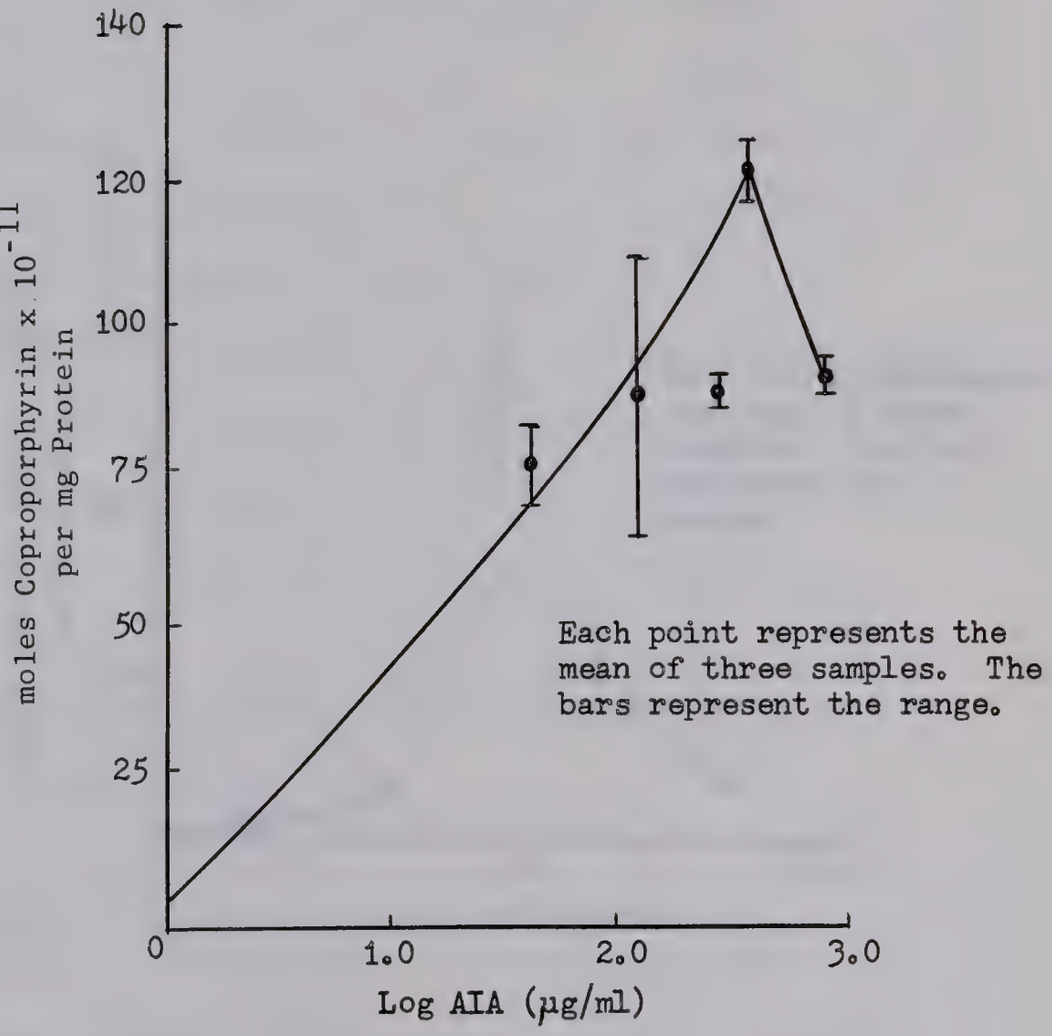


FIGURE 13 RELATIONSHIP BETWEEN DOSE OF AIA AND PORPHYRIN ACCUMULATION

obtained are shown in figures 12, 13 and 14.

ii. Effect of UDPGA on Porphyria-Induction Caused by Pregnanolone

The cell colonies were prepared in the usual manner

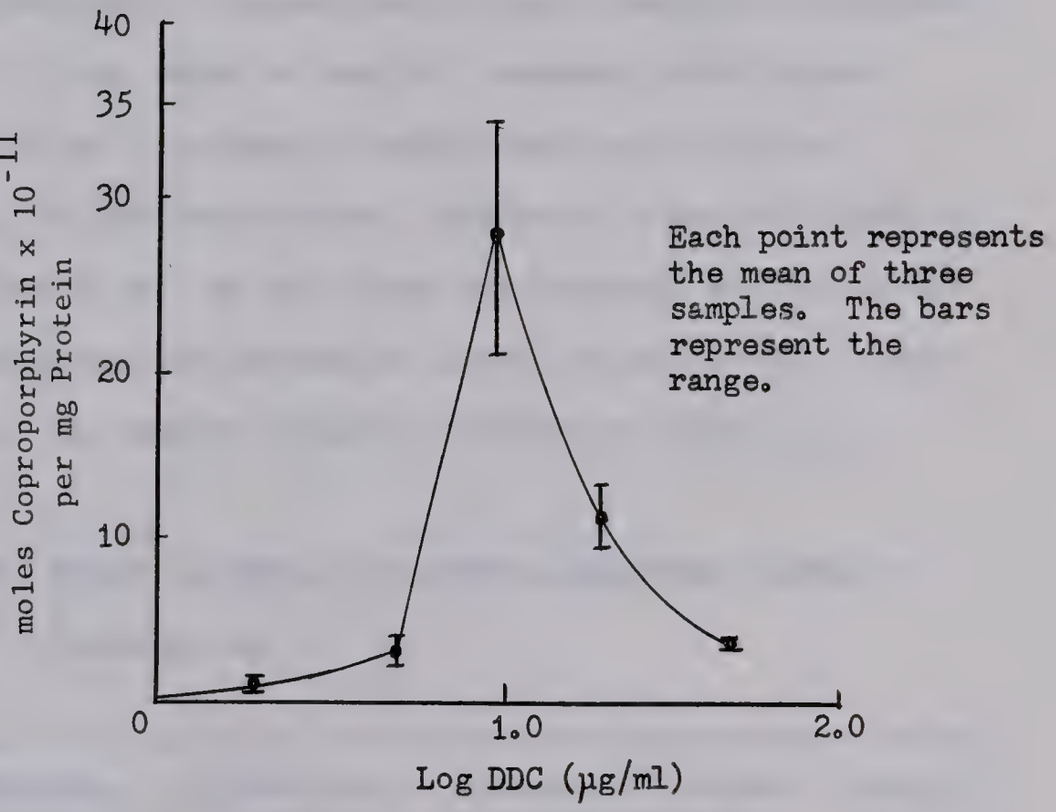


FIGURE 14 RELATIONSHIP BETWEEN DOSE OF DDC AND PORPHYRIN ACCUMULATION

and allowed to incubate for 24 hr. The culture medium was then removed and replaced by fresh medium (4 ml). A dose of pregnanolone (8 µg/ml medium) was chosen from the pregnanolone dose-response curve as an appropriate dose to be used in the subsequent experiments. Pregnanolone (32 µg) dissolved in propylene glycol (5λ) was added to each of 6 separate petri dishes. UDPGA (4000 µg) dissolved in Earle's Medium (0.1 ml) was added to 3 of the petri dishes. The petri dishes were then incubated for 24 hr. At this time, the porphyrin content of the cells and media were determined as well as the protein content per dish. The results obtained are shown in table III.

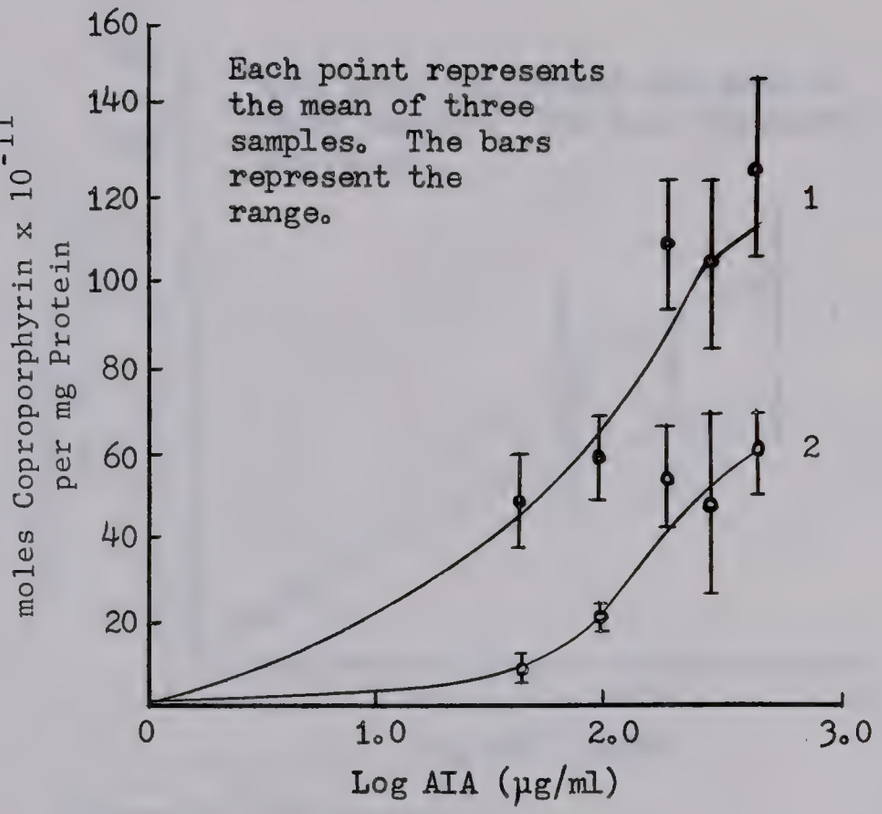
TABLE III EFFECT OF UDPGA ON PORPHYRIA-INDUCTION CAUSED BY PREGNANOLONE

µg Pregnanolone per ml medium	µg UDPGA per ml medium	moles x 10^{-11} copro-porphyrin produced per mg protein	Range (±)
0	0	2.21*	-
8	0	83.08*	10.76
0	1000	1.64*	-
8	1000	31.82*	23.34

* Each value represents the mean of three samples.

iii. Effect of UDPGA on Porphyria-Induction Caused by AIA

The cell colonies were prepared in the usual manner and allowed to incubate for 24 hr. The culture medium was then

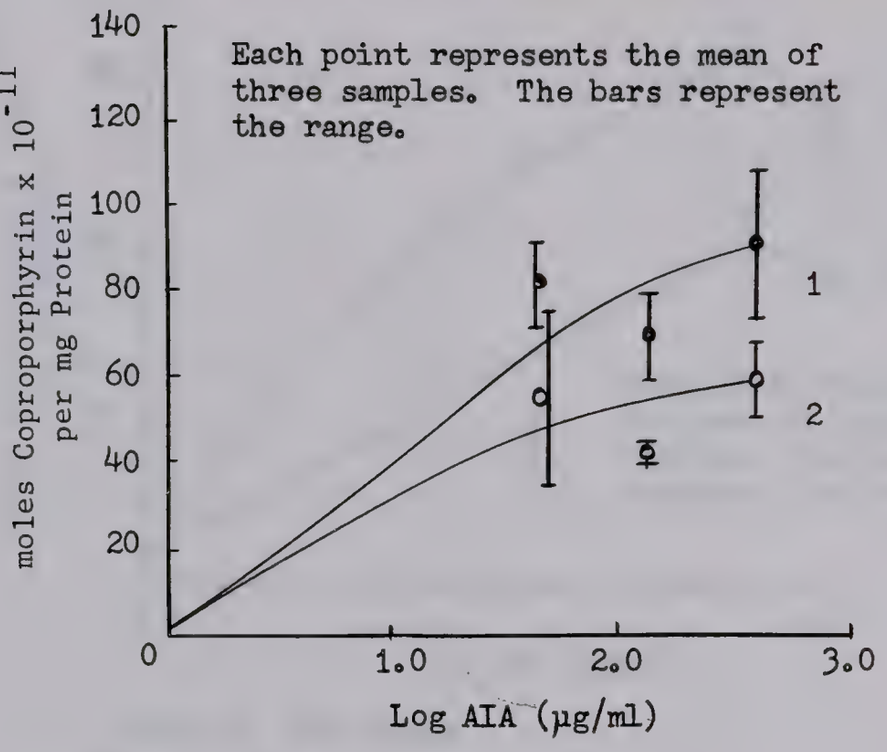


Curve 1: AIA alone

Curve 2: AIA + UDPGA

FIGURE 15A EFFECT OF UDPGA ON PORPHYRIA-INDUCTION CAUSED BY AIA

removed and replaced with fresh medium (4 ml). The doses of AIA chosen from the AIA dose-response curve varied from 50 - 500 µg/ml medium. The effect of UDPGA on each concentration of AIA was tested as follows: AIA dissolved in ethanol (95%; 1 - 5 λ) was added to each of 6 petri dishes. UDPGA (4000 µg) dissolved in Earle's Medium (0.1 ml) was added to 3 of the petri dishes. The dishes were then incubated for 24 hr at which time the porphyrin content of the cells and supernatant was determined, as well as

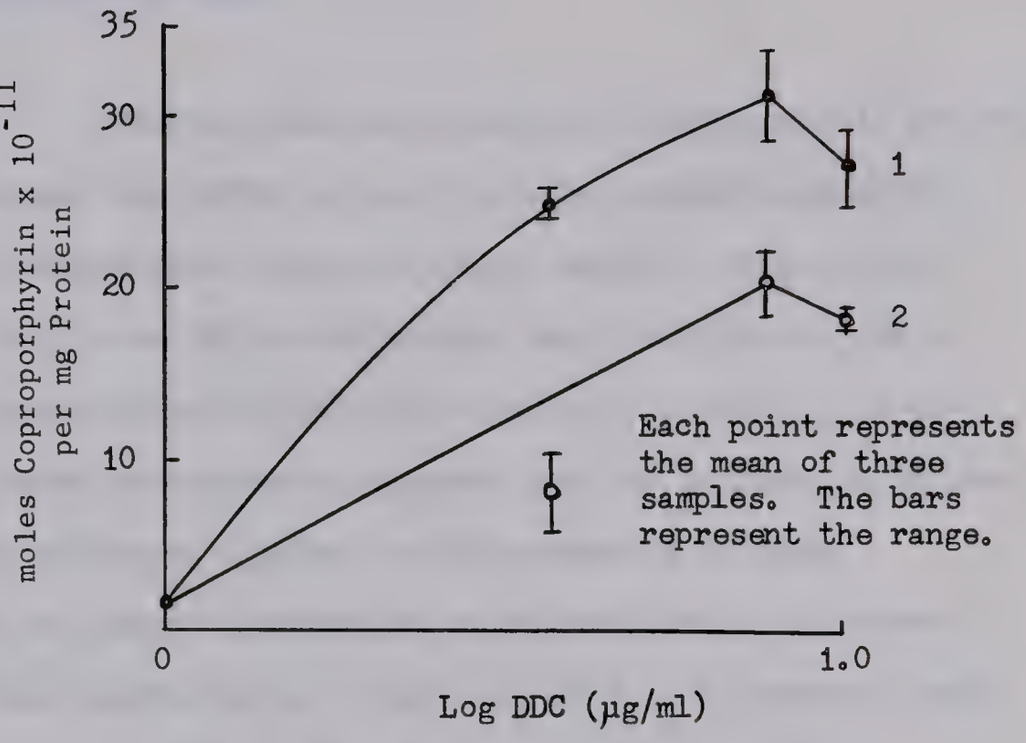


Curve 1: AIA alone

Curve 2: AIA + UDPGA

FIGURE 15B EFFECT OF UDPGA ON PORPHYRIA-INDUCTION CAUSED BY AIA

the protein content. The results are shown in Figures 15A and 15B. Two other experiments were performed to study the effect of UDPGA on porphyria-induction caused by AIA, but the maximum amount of coproporphyrin produced per mg protein was in both cases less than 25 moles x 10^{-11}. As this value was well below the amount of porphyrin usually obtained, it was concluded that the chick embryo liver cell culture system was not responding to the inducing chemical, and the results were therefore discarded.



Curve 1: DDC alone

Curve 2: DDC + UDPGA

FIGURE 16 EFFECT OF UDPGA ON PORPHYRIA-INDUCTION CAUSED BY DDC

iv. Effect of UDPGA on Porphyria-Induction Caused by DDC

For this experiment, concentrations of 4, 8, and 10 µg DDC/ml medium were chosen from the DDC dose-response curve. DDC was dissolved in 95% ethanol (1 - 5 λ). The procedure followed was identical to that followed for AIA. The results obtained are plotted in Fig. 16.

Discussion of the Results

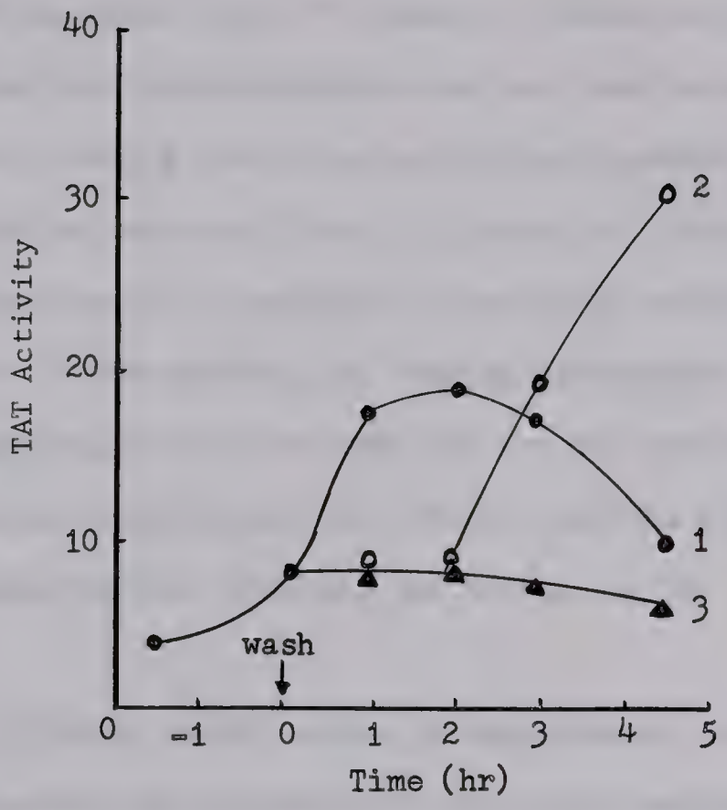
From the results in Table III and Figures 15 and 16, it can be seen that UDPGA not only inhibits porphyria-induction caused by pregnanolone, but also by AIA and DDC. This was not expected as AIA and DDC do not contain any of the groups which can be glucuronidated (Dutton, 1966: Table II, p. 460). On the basis of these experiments it appears that the explanation offered by Granick and Kappas (1967a) for the mechanism of UDPGA inhibition of porphyria-induction by pregnanolone is incorrect. However, the results are not clear cut since it is possible that AIA and DDC are converted by the liver cells to metabolites containing groupings which would allow them to be glucuronidated. It was thus necessary to carry out further experiments to define the mechanism of action of UDPGA. These experiments are reported in the next chapter of this thesis.

CHAPTER IV STUDIES OF THE MECHANISM OF ACTION OF UDPGA ON DRUG-INDUCED PORPHYRIN FORMATION

Introduction

In Chapter III of this thesis, it was shown that UDPGA inhibits porphyria-induction caused by AIA and DDC, as well as by pregnanolone. As AIA and DDC do not have groupings that can combine with glucuronic acid, resulting in an inactive glucuronidated derivative, it was decided to investigate the mechanism by which UDPGA acts to inhibit porphyria-induction caused by these chemicals.

Peterkofsky and Tomkins (1968) studied the steroid-induced accumulation of tyrosine-aminotransferase (TAT) mRNA in an established line of cultured HTC cells derived from Morris rat hepatoma 7288c. In their first series of experiments, they were interested to see if the steroid inducer stimulated the accumulation of TAT mRNA in the absence of protein synthesis. Using cycloheximide (CH) as an inhibitor of protein synthesis and the glucocorticoid dexamethasome phosphate (Dex) as the inducing steroid, they carried out their experiments in two steps. The cells were first preincubated for 1.5 - 2 hr in media containing CH or CH in combination with Dex. After washing out the drugs from the cells, the cells were then reincubated for 3 - 5 hr in the presence or absence of Dex. At various time intervals TAT activity was determined. Preincubation of cells with a combination of Dex and CH led, after removal of these drugs by washing, to a rapid increase in TAT activity upon

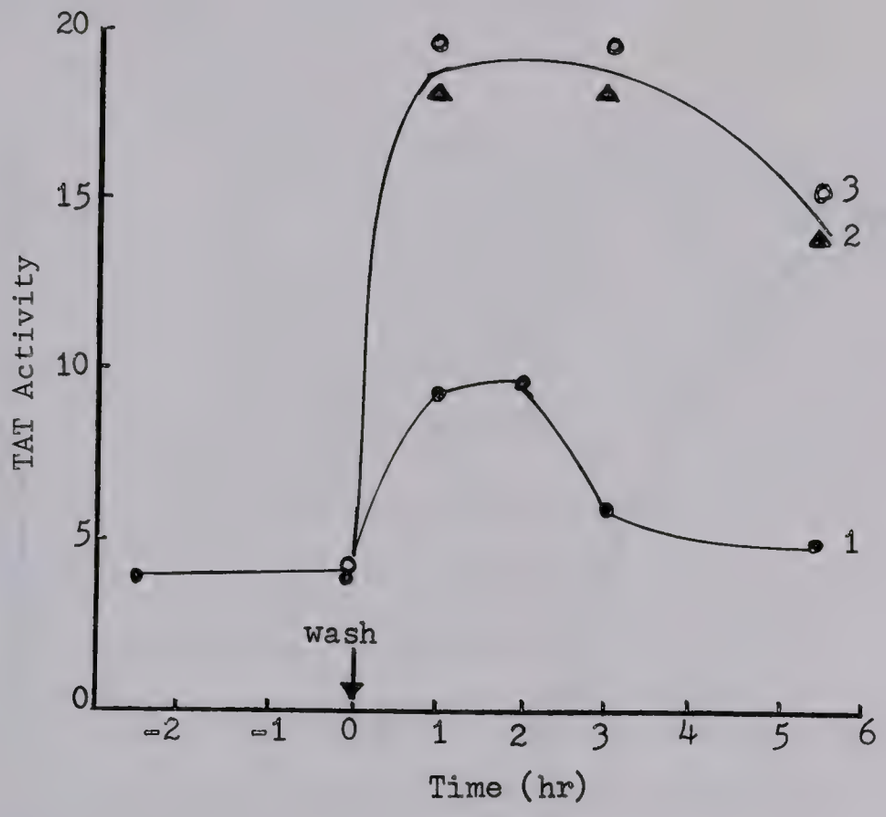


	Preincubation	Reincubation
Curve 1:	CH + Dex	No Addition
Curve 2:	CH	Dex
Curve 3:	CH	No Addition

FIGURE 17 TAT ACTIVITY AFTER PREINCUBATION OF HTC CELLS WITH OR WITHOUT VARIOUS ADDITIONS OF DEXAMETHASONE (Dex) AND CYCLOHEXIMIDE (CH). (From Peterkofsky and Tomkins, 1968).

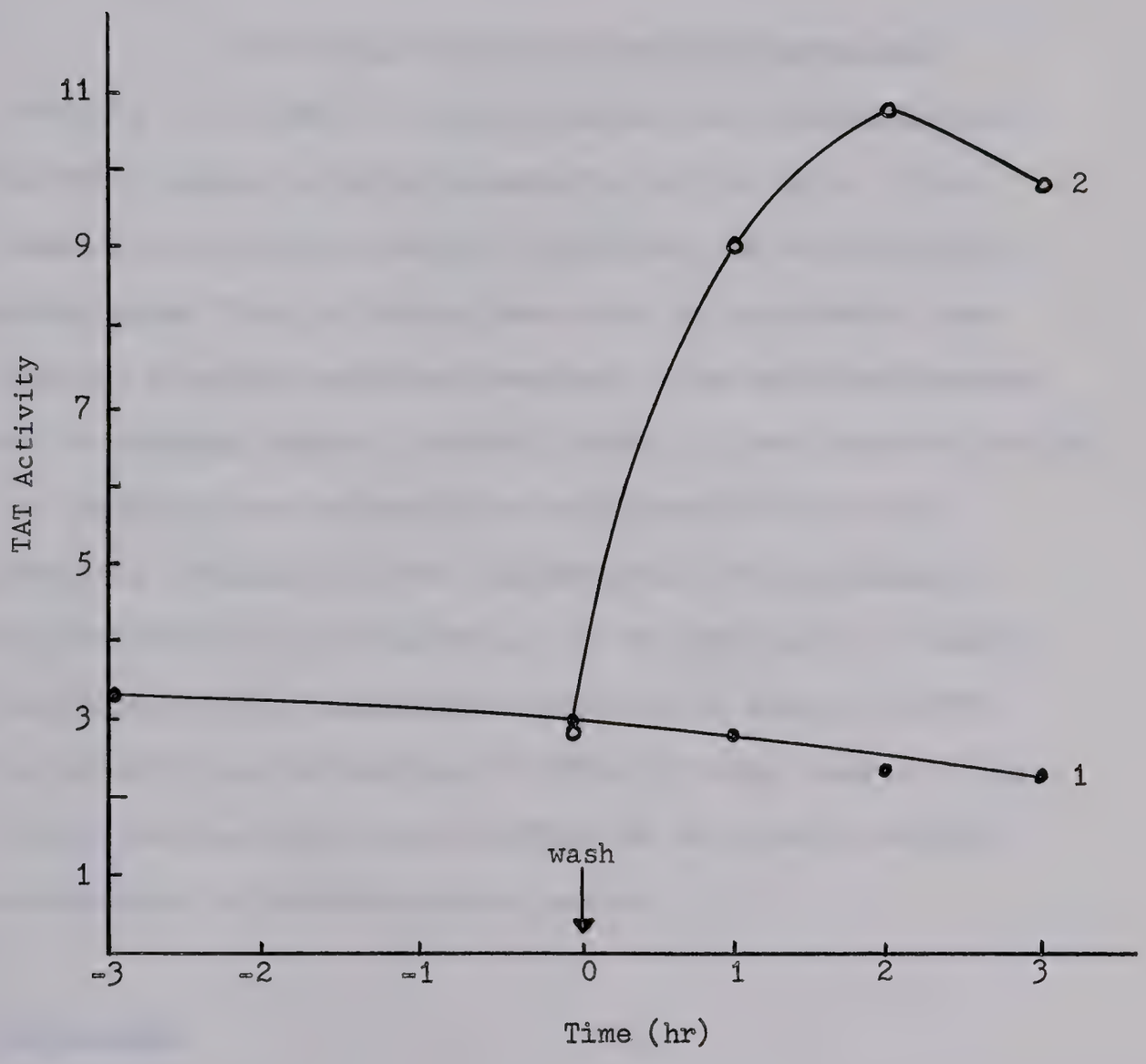
reincubation of the cells (Fig. 17, curve 1). When the cells had been preincubated with cycloheximide alone and then reincubated in the presence of Dex, a two hr lag period was observed before TAT induction was noted (Fig. 17, curve 2). Preincubation with CH alone resulted in a negligable rise in TAT activity (Fig. 17, curve 3). Peterkofsky and Tomkins interpreted these results by suggesting that an intermediate for TAT synthesis had accumulated during the preincubation period under the influence of Dex even though protein synthesis was blocked by CH.

In their second series of experiments, they added different combinations of Actinomycin D (Act), CH, and Dex to the cell culture during the preincubation period, and after washing the cells. If Act (Fig. 18, curve 1) was added during the preincubation period with Dex and CH the subsequent rise in TAT activity was greatly reduced. Addition of Act after a preincubation with Dex and CH (Fig. 18, curve 3) did not inhibit the rise of TAT activity. Thus, Dex acts in the preincubation period when protein synthesis is inhibited to cause an accumulation of mRNA required for the synthesis of TAT. The increase in TAT activity after preincubation with Dex and CH requires new protein synthesis since the rise could be prevented if CH was added after washing (Fig. 19). In view of the successful application of this method to investigate steroid-induced increase of TAT activity by the above authors, we decided to follow a similar series of experiments in our investigations of the mechanism of action of UDPGA on porphyrin-induction by



	Preincubation	Reincubation
Curve 1:	Act., CH, Dex	No Addition
Curve 2:	CH, Dex	No Addition
Curve 3:	CH, Dex	Act

FIGURE 18 THE EFFECT OF ACTINOMYCIN D (Act) ADDED DURING PREINCUBATION ON SUBSEQUENT INDUCTION OF TAT (From Peterkofsky and Tomkins, 1968).



	Preincubation	Reincubation
Curve 1:	CH, Dex	CH
Curve 2:	CH, Dex	No Addition

FIGURE 19 THE INHIBITION OF INDUCTION AFTER PREINCUBATION WITH STEROID AND CYCLOHEXIMIDE (CH) BY READDITION OF CYCLOHEXIMIDE DURING THE SECOND INCUBATION (From Peterkofsky and Tomkins, 1968).

pregnanolone, AIA, and DDC.

According to Granick (1966), AIA stimulates synthesis of the mRNA for δ-aminolevulinic acid synthetase and therefore causes porphyrin accumulation in the cells. After removal of AIA from the cells, a significant rise in porphyrin accumulation (Fig. 20) takes place which is considerably less than the rise that would have occurred in the continued presence of the inducing chemical (Granick, 1966). It was therefore decided to investigate what effect UDPGA would have on the rise in porphyrin accumulation after the porphyria-inducing chemical had been washed out of the cells. If the hypothesis of Granick and Kappas (1967a) regarding the mechanism of action of UDPGA is correct, then the addition of UDPGA following removal of the drug by washing should have no effect on the rise in porphyrin accumulation in the reincubation period.

Experimental

A. AIA

i) Procedure for Removal of AIA from the Cells:

In the above experiment (Fig. 20) following the initial incubation Granick washed the cells 3 times with fresh medium (4 ml) at 5 min intervals. He assumed without evidence

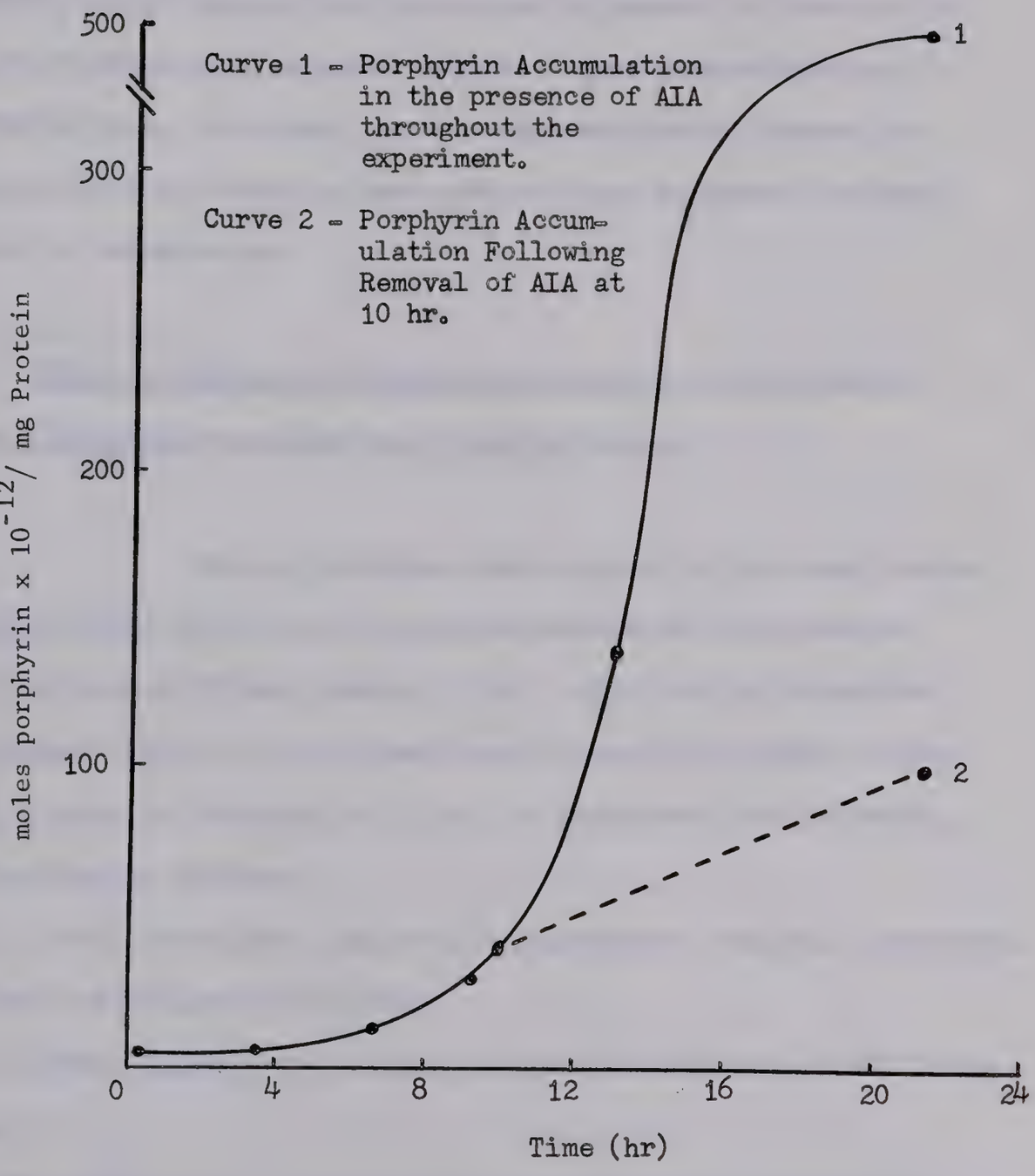


FIGURE 20 RATE OF PORPHYRIN FORMATION IN CHICK EMBRYO LIVER CELLS AFTER INDUCTION WITH AIA (300 µg/ml) AND AFTER REMOVAL OF AIA (From Granick, 1966).

that this procedure sufficed to remove all the AIA (Granick, 1966). Schneck (1969) checked this procedure for removal of the drug by a method which will be described later using pregnanolone as inducing drug. He showed that one wash sufficed to remove the drug. For this reason we have used one wash to remove the drug prior to reincubation.

ii) Effect of UDPGA on Porphyrin Accumulation in Chick Embryo Liver Cells Following Removal of AIA by Washing

The cell colonies were prepared in the usual manner and incubated for 24 hr. The culture medium was then removed and replaced with fresh medium (4 ml). AIA (1200 µg) dissolved in ethanol (95%; 2 λ) was then placed in each of 20 petri dishes and allowed to incubate for 12 hr. At this time, the following procedure was followed:

a) 5 petri dishes were analyzed for supernatant and cell porphyrins, as well as the protein content;

b) 5 petri dishes were returned to the incubator for an additional 5 hr;

c) The medium from 5 petri dishes was removed and retained for porphyrin analysis. The cells were washed once with fresh medium (4 ml) for 0.17 hr and then reincubated for 5 hr;

d) The medium from 5 petri dishes was removed and retained for porphyrin analysis. The cells were washed once with fresh medium (4 ml) for 0.17 hr, UDPGA (4000 µg) dissolved in Earle's Medium

(0.1 ml) and fresh medium (4 ml) was added to each petri dish, and the cells were allowed to reincubate for 5 hr;

e) At the end of the 12 hr reincubation period, the petri dishes were analyzed for supernatant and cell porphyrins, as well as protein content. The results are shown in Table IV and Fig. 21.

B. Pregnanolone

i. Procedure for the Removal of Pregnanolone from the Cells:

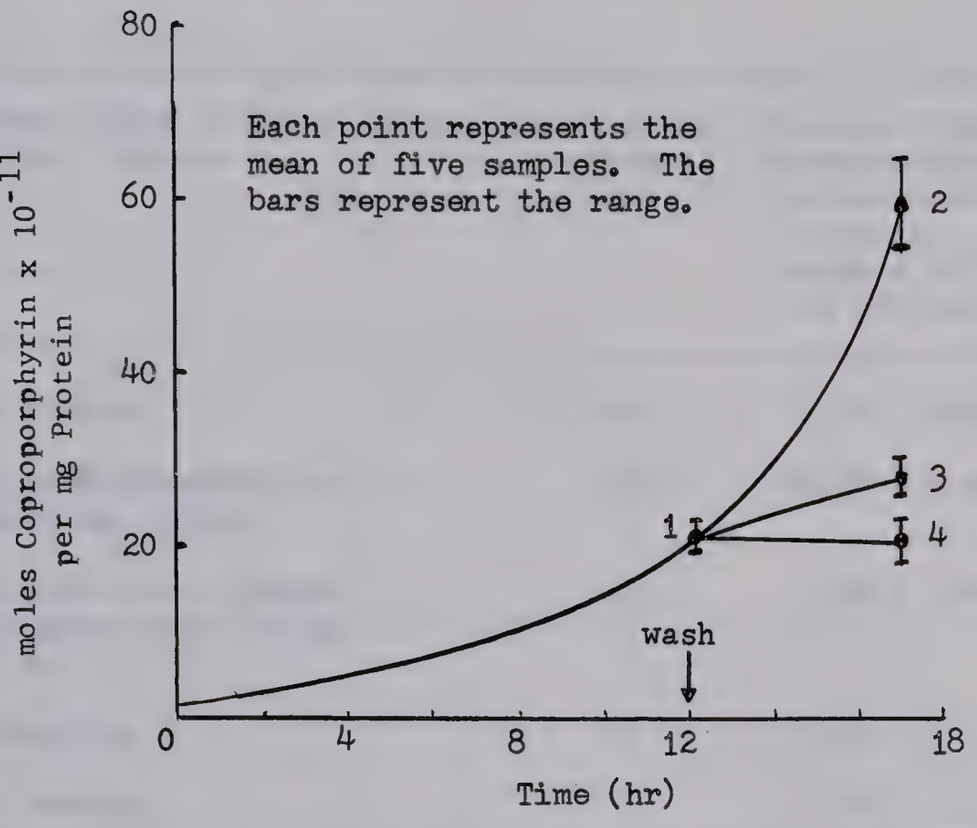
In order to determine the number of washings required to remove pregnanolone from the cells we have checked the initial medium and subsequent washings for the presence of pregnanolone. This was done by the following procedure of adding the solutions to untreated chick embryo liver cells and estimating the presence of pregnanolone by the porphyrin accumulation induced after a reincubation period. The cell colonies were prepared in the usual manner and allowed to incubate for 24 hr. The culture medium was then removed and replaced by fresh medium (4 ml). Pregnanolone (32 µg) dissolved in propylene glycol (5 λ) was added to 3 of the petri dishes, and these were allowed to incubate for 12 hr. At this time, the supernatant was removed from the petri dishes and placed in 3 other petri dishes containing untreated cells. Fresh culture medium (4 ml) was then placed in the origional petri dishes, and after an additional 10 min, this medium was in turn removed and placed in 3 petri dishes

TABLE IV EFFECT OF UDPGA ON PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER CELLS FOLLOWING REMOVAL OF AIA BY WASHING *

Solutions Added to Chick Embryo Liver Cell Cultures	Duration of Treatment (hr)	Average Total Coproporphyrin Accumulation ± Range (moles x 10^{-11}/ mg Protein)
Normal Medium	17	0.90
Normal Medium containing AIA (1200 µg)	12	21.50 ± 2.58
Normal Medium containing AIA (1200 µg)	17	57.17 ± 5.17
i. Medium containing AIA (1200 µg)	12	
ii. Fresh Medium	0.17	
iii. Fresh Medium	5	29.38 ± 2.13 **
i. Medium containing AIA (1200 µg)	12	
ii. Fresh Medium	0.17	
iii. Fresh Medium containing UDPGA (4000 µg)	5	21.77 ± 4.36 **

* Monolayer cultures of liver cells were grown on petri dishes as previously described. After 24 hr of growth the cells were treated as described in the table and the porphyrin content of cells and media determined. Each solution was tested in 5 separate petri dishes.

** For these cells the porphyrin content of the media after 12 hr of growth in the presence of AIA was determined and added to that of the cells and media following a further 5 hr of incubation.



	Preincubation	Reincubation
Curve 1:	AIA	Stopped at 12 hr
Curve 2:	AIA	AIA
Curve 3:	AIA	No Addition
Curve 4:	AIA	UDPGA

FIGURE 21 EFFECT OF UDPGA ON PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER CELLS FOLLOWING REMOVAL OF AIA BY WASHING

TABLE V DETECTION OF PREGNANOLONE IN THE MEDIA AND WASHINGS OF CHICK EMBRYO LIVER CELLS BY MEANS OF A BIOASSAY PROCEDURE *

Solutions Added to Chick Embryo Liver Cell Culture	Duration of Treatment (hr)	Average Total Coproporphyrin Accumulation ± Range (moles x 10^{-11}/ mg Protein)
Normal Medium	24	0.98 ± 0.09
Normal Medium containing Pregnanolone (32 µg)	12	26.23 ± 2.94
Medium from Cells Treated with Pregnanolone (32 µg) for 12 hr	12	7.30 ± 1.06
First Washing	12	1.96 ± 0.52
Second Washing	12	1.36 ± 0.10

* Monolayer cultures of liver cells were grown on petri dishes as previously described. After 24 hr of growth Pregnanolone (32 µg) was added to one half of the cultures while the other half received none. Following an incubation period of 12 hr the medium from pregnanolone treated cells was removed for testing. The cells were washed at 10 min intervals with fresh medium (4 ml) and these washings were removed for testing as follows: The original media and washings were added individually to fresh monolayers of chick embryo liver cells which had been allowed to grow 24 hr and had had their medium removed. These cells were then incubated a further 12 hr after which the porphyrin content of the media and cells was determined. Each solution was tested in three separate petri dishes.

containing untreated cells. This process of washing the initially drug-treated cells with fresh medium and then transferring the medium to petri dishes containing untreated cells was repeated. The cells were then allowed to incubate for 12 hr, at which time the porphyrin content of the cells and supernatant, as well as the protein content of the dishes was determined. The results are shown in Table V.

ii. Effect of UDPGA on Porphyrin Accumulation in Chick Embryo Liver Cells Following the Removal of Pregnanolone by Washing

The method followed was the same as that outlined for the effect of UDPGA on porphyrin accumulation following removal of AIA by washing except that the reincubation period was 12 hr. The results obtained are shown in Table VI.

C. DDC

i. Procedure for the Removal of DDC from the Cells

In order to determine the number of washings required to remove DDC from cells after an incubation period of 8 hr, the same procedure was used as outlined for pregnanolone. The results are shown in Table VII.

ii. Effect of UDPGA on Porphyrin Accumulation in Chick Embryo Liver Cells Following Removal of DDC by Washing

TABLE VI EFFECT OF UDPGA ON PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER CELLS FOLLOWING REMOVAL OF PREGNANOLONE BY WASHING *

Solutions Added to Chick Embryo Liver Cell Cultures	Duration of Treatment (hr)	Average Total Coproporphyrin Accumulation ± Range (moles x 10^{-11}/ mg Protein)
Normal Medium	24	1.05 ± 0.10
Normal Medium containing Pregnanolone (32 µg)	12	3.58 ± 0.35
Normal Medium containing Pregnanolone (32 µg)	24	56.27 ± 7.95
i. Medium containing Pregnanolone (32 µg)	12	
ii. Fresh Medium	0.17	
iii. Fresh Medium	12	15.56 ± 1.42 **
i. Medium containing Pregnanolone (32 µg)	12	
ii. Fresh Medium	0.17	
iii. Fresh Medium containing UDPGA (4000 µg)	12	14.01 ± 0.66 **

* Monolayer cultures of liver cells were grown on petri dishes as previously described. After 24 hr of growth the cells were treated as described in the table and the porphyrin content of cells and media determined. Each solution was tested in 5 separate petri dishes.

** For these cells the porphyrin content of the media after 12 hr of growth in the presence of pregnanolone was determined and added to that of the cells and media following a further 12 hr of incubation.

TABLE VII DETECTION OF DDC IN THE MEDIA AND WASHINGS OF CHICK EMBRYO LIVER CELLS BY MEANS OF A BIOASSAY PROCEDURE *

Solutions Added to Chick Embryo Liver Cell Culture	Duration of Treatment (hr)	Average Total Coproporphyrin Accumulation ± Range (moles x 10^{-11}/ mg Protein)
Normal Medium	17	1.05 ± 0.14
Normal Medium containing DDC (32 µg)	8	5.74 ± 0.98
Media from Cells Treated with DDC (32 µg) for 8 hr	9	13.40 ± 0.63
First Washing	9	1.43 ± 0.40
Second Washing	9	1.31 ± 0.28
Third Washing	9	1.0 ± 0.17

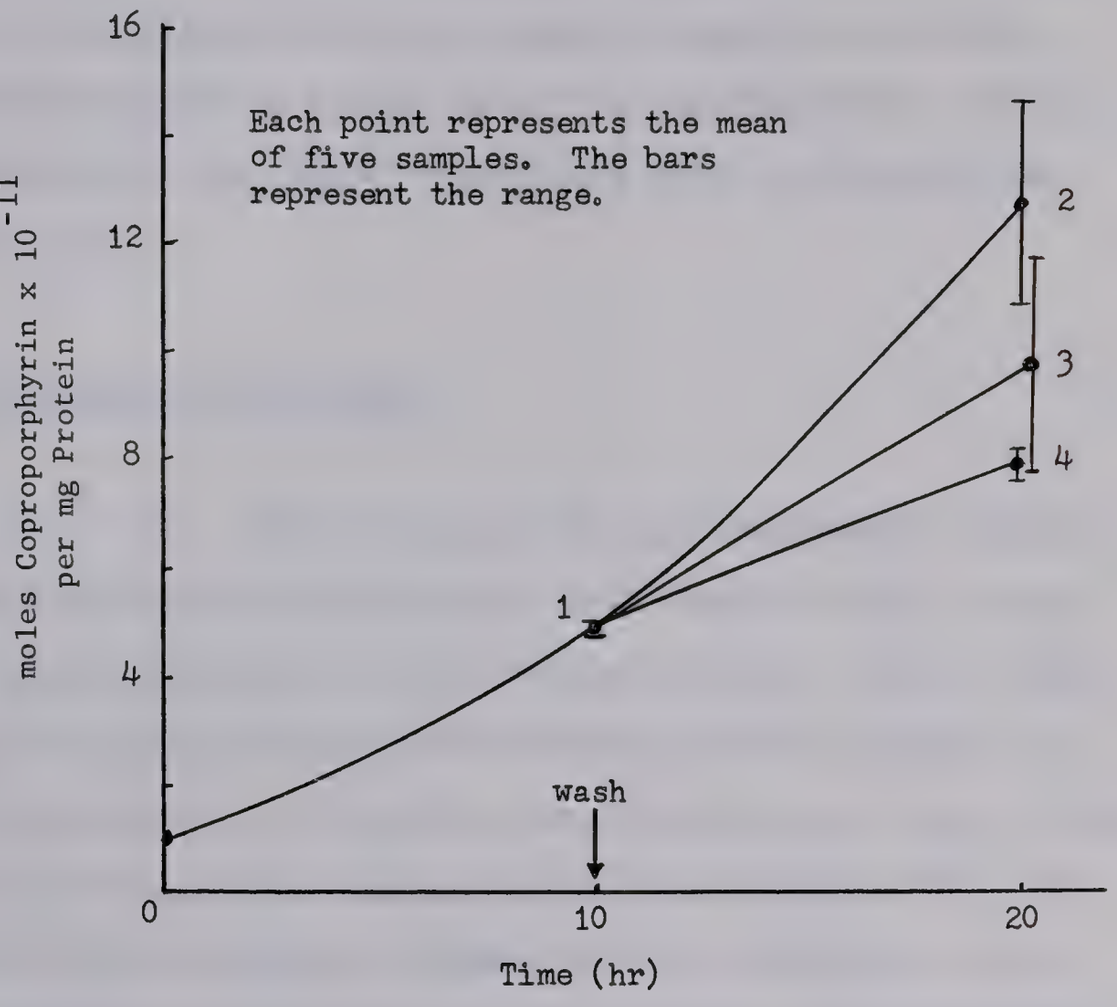
* Monolayer cultures of liver cells were grown on petri dishes as previously described. After 24 hr of growth DDC (32 µg) was added to one half of the cultures while the other half received none. Following an incubation period of 8 hr the media from DDC treated cells was removed for testing. The cells were washed at 10 min intervals with fresh medium and these washings were added individually to fresh monolayers of chick embryo liver cells which had been allowed to grow 24 hr and had had their media removed. These cells were then incubated a further 9 hr after which the porphyrin content of media and cells was determined. Each solution was tested in five separate petri dishes.

TABLE VIII EFFECT OF UDPGA ON PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER CELLS FOLLOWING REMOVAL OF DDC BY WASHING *

Solutions Added to Chick Embryo Liver Cell Culture	Duration of Treatment (hr)	Average Total Coproporphyrin Accumulation ± Range (moles x 10^{-11}/ mg Protein)
Normal Medium	20	1.05 ± 0.14
Normal Medium containing DDC (32 µg)	10	4.96 ± 0.30
Normal Medium containing DDC (32 µg)	20	12.64 ± 2.48
i. Medium containing DDC (32 µg)	10	
ii. Fresh Medium	0.17	
iii. Fresh Medium	10	9.57 ± 2.58 **
i. Medium containing DDC (32 µg)	10	
ii. Fresh Medium	0.17	
iii. Fresh Medium containing UDPGA (4000 µg)	10	7.78 ± 0.40 **

* Monolayer cultures of liver cells were grown in petri dishes as previously described. After 24 hr of growth the cells were treated as described in the table and the porphyrin content of cells and media determined. Each solution was tested in 5 separate petri dishes.

** For these cells the porphyrin content of the media after 10 hr of growth in the presence of DDC was determined and added to that of the cells and media following a further 10 hr of incubation.



	Preincubation	Reincubation
Curve 1:	DDC	Stopped at 10 hr
Curve 2:	DDC	DDC
Curve 3:	DDC	No Addition
Curve 4:	DDC	UDPGA

FIGURE 22 EFFECT OF UDPGA ON PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER CELLS FOLLOWING REMOVAL OF DDC BY WASHING

The method followed was the same as that outlined for the effect of UDPGA on porphyrin accumulation following removal of AIA by washing except that the reincubation period was 10 hr. The results obtained are shown in Table VIII and Fig. 22.

Discussion of the Results

After adding AIA to cells considerable porphyrin accumulation was observed after 12 hr (Table IV; Fig. 21) and this increased markedly over the next 5 hours. Removal of AIA at 12 hours by washing followed by reincubation resulted in a significant rise in porphyrin accumulation after 5 hours. These observations are in agreement with those of Granick (Fig. 20). This rise in porphyrin accumulation upon reincubation is not prevented by Actinomycin D (Schneck, 1969). Since Actinomycin D prevents drug-induced porphyrin accumulation it follows that the rise in porphyrin accumulation during the reincubation period is not due to residual drug in the cells. Rather it must be due to the mRNA for δ-aminolevulinic acid synthetase or δ-amino-levulinic acid synthetase itself or both as suggested by Granick. The results in Table IV and Fig. 21 show clearly that UDPGA prevents the rise in porphyrin accumulation during the reincubation period. Since the drug is not present during this time it is clear that UDPGA is not inhibiting porphyrin accumulation by enhancing the glucuronidation and inactivation of drug.

The results with DDC while apparently similar to those with AIA were not sufficiently clear cut ($0.10 > P > 0.05$) to allow a decision as to whether UDPGA significantly lowered the rise in porphyrin accumulation observed in the reincubation period. It is likely that a series of further experiments will clarify this point.

The results observed in the pregnanolone experiment (Table VI) show that UDPGA does not affect the rise in porphyrin accumulation in the reincubation period when pregnanolone has been removed. These results are in accordance with the interpretation of Granick viz., that UDPGA enhances the glucuronidation and hence inactivation of pregnanolone.

These results are difficult to explain since it was anticipated that the effects of UDPGA on porphyrin accumulation in the reincubation period would have been the same irrespective of the drug used. However, it is conceivable that the mechanism of steroid-induced porphyrin biosynthesis is not the same as AIA and DDC induced porphyrin biosynthesis. This possibility has to be seriously considered in view of the fact that steroids induce in immature red cells as well as liver cells while AIA and DDC induce only in liver cells. It is clear from the results obtained with AIA that UDPGA is able to inhibit drug-induced porphyrin accumulation by mechanisms other than enhanced glucuronidation of a drug. Recently Hickman et al. (1968) have provided evidence leading to the suggestion that the inhibitory effects of carbohydrates on the induction of

δ-aminolevulinic acid synthetase are due to an interference by carbohydrates with the coding of mRNA for δ-aminolevulinic acid synthetase. Such a mechanism could explain the inhibition we have observed with UDPGA using AIA as the inducer.

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